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Date: October 18, 2000

Docket No.: 1422-0442P

Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

This is a Request for filing a  continuation  divisional  
 continuation-in-part application under 37 C.F.R. § 1.53(b) of  
pending prior Application No. 08/952,089 filed on November 10,  
1997, the entire contents of which are hereby incorporated by  
reference,  
by

SAGAWA, Hiroaki; UENO, Harumi; OSHIMA, Atsushi; and KATO,  
Ikunoshin

for

PLASMID

1.  Enclosed is an application consisting of specification, claims, declaration and drawings/photographs (if applicable).
2.  The filing fee has been calculated as follows:

			LARGE ENTITY	SMALL ENTITY
	BASIC FEE		\$710.00	\$355.00
	NUMBER FILED	NUMBER EXTRA	RATE FEE	RATE FEE
<b>TOTAL CLAIMS</b>	9-20 =	0	x 18 = \$0.00	x 9 = \$0.00
<b>INDEPENDENT CLAIMS</b>	6-3 =	3	x 80 = \$240.00	x 40 = \$0.00
<input type="checkbox"/> MULTIPLE DEPENDENT CLAIMS PRESENTED			+ \$270.00	+ \$135.00
		<b>TOTAL</b>	<b>\$950.00</b>	<b>\$0.00</b>

3.  A check in the amount of \$950.00 to cover the filing fee and recording fee (if applicable) is enclosed.
4.  Please charge Deposit Account No. 02-2448 in the amount of \$0.00. A triplicate copy of this request is enclosed.
5. Amend the specification by inserting before the first line thereof the following:
- a.  --This application is a  continuation  divisional  continuation-in-part of co-pending Application No. 08/952,089, filed on November 10, 1997, the entire contents of which are hereby incorporated by reference.--
- b.  --This application is a  continuation  divisional  continuation-in-part of co-pending Application No. 08/952,089, filed on November 10, 1997. Application No. 08/952,089 is the national phase of PCT International Application No. PCT/JP97/00748 filed on March 10, 1997 under 35 U.S.C. § 371. The entire contents of each of the above-identified applications are hereby incorporated by reference.--
6.  Enclosed is/are twenty-six (26) sheet(s) of formal drawings and/or photographs.
7.  A statement claiming small entity status was filed in prior Application No. 08/952,089 on \_\_\_\_\_. See the attached copy of the statement claiming small entity status.

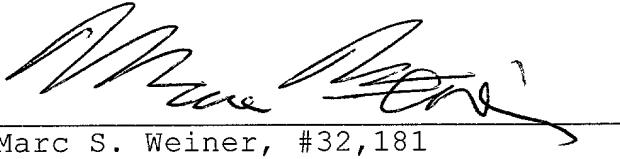
8.  The prior application is assigned to Takara Shuzo Co., Ltd..
9.  A Preliminary Amendment is enclosed.
- 10a.  Priority of Application No(s). \_\_\_\_\_ filed in \_\_\_\_\_ on \_\_\_\_\_ is/are claimed under 35 U.S.C. § 119. See attached copy of the Letter claiming priority filed in the prior application on \_\_\_\_\_.
- 10b.  Priority of International Appln. PCT/JP97/00748 filed on March 10, 1997 under the Patent Cooperation Treaty and Japanese Application No. 8-85801 and 8-208897 filed in Japan on March 13, 1996 and July 18, 1996, respectively under 35 U.S.C. § 119 are hereby reclaimed.
11.  An Information Disclosure Statement and PTO-1449 form(s) are attached hereto for the Examiner's consideration.
12.  Address all future communications to:  
BIRCH, STEWART, KOLASCH & BIRCH, LLP  
P.O. Box 747  
Falls Church, VA 22040-0747  
Telephone: (703) 205-8000  
or  
Customer No. 2292
13.  An extension of time for \_\_\_\_\_( ) month(s) until has been submitted in parent Application No. 08/952,089 in order to establish co-pendency with the present application.
14.  Also enclosed herewith is the following:  
Copies of receipts for deposits of microorganisms.  
\_\_\_\_\_  
\_\_\_\_\_

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By

  
Marc S. Weiner, #32,181

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MSW/MAA/csp  
1422-0442P

Attachments

(Rev. 09/29/2000)

PATENT  
1422-0442P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant: Hiroaki SAGAWA et al.

Appl. No.: Divisional of 08/952,089 Group: Unassigned

Filed: October 18, 2000 Examiner: Unassigned

For: PLASMID

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents  
Washington, DC 20231

October 18, 2000

Sir:

The following preliminary amendments and remarks are respectfully submitted in connection with the above-identified application.

AMENDMENTS

IN THE SPECIFICATION:

Page 19

Line 22, please insert --pMS434 contains in part DNA having the DNA sequence identified as SEQ ID NO:31. pMSP6L contains in part DNA having the DNA sequence identified as SEQ ID NO:32. --

Line 24, please insert --pMS434 contains in part DNA having the DNA sequence identified as SEQ ID NO:31. pMSP6L contains in part

DNA having the DNA sequence identified as SEQ ID NO:32. pMSP60 contains in part DNA having the DNA sequence identified as SEQ ID NO:33. --

Please replace pages 82-100 of the specification with the Substitute Sequence Listing enclosed herewith. Please renumber the remaining pages, beginning with the claims, consecutively from page 98 of the Sequence Listing.

IN THE CLAIMS:

Please cancel claim 1-19 without prejudice or disclaimer of the subject matter contained therein.

Please amend the claims as follows:

Claim 20, A method for isolating a desired gene, characterized in that [the] a plasmid vector comprising a promoter sequence to control an expression of a desired gene, said promoter sequence being recognized by an RNA polymerase not inherent to a host, and a replication origin for increasing a copy number by induction with an exogenous factor.

Claim 28, line 2, please change "any one of claims 25 to 27" to  
--claim 25--.

**REMARKS**

Enclosed herewith in full compliance with 37 C.F.R. 1.821-1.825 is a Substitute Sequence Listing to be inserted into the specification as indicated above. The Substitute Sequence Listing in no way introduces new matter into the specification.

In further compliance with 37 C.F.R. 1.821-1.825 please transfer the disk copy of the Substitute Sequence Listing named file 1422-319.sub, filed on July 17, 1998 in the parent application to the present application. The disk copy of the Substitute Sequence Listing, file 1422-319.sub, is identical to the paper copy submitted herewith, except that it lacks formatting.

Entry of the above amendments is earnestly solicited. An early and favorable first action on the merits is earnestly solicited.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact MaryAnne Armstrong, Ph.D. (Reg. 40,069) at the telephone number of the undersigned below.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By   
Marc S. Weiner, #32,181

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(Rev. 04/19/2000)

DESCRIPTION

PLASMID

5       TECHNICAL FIELD

The present invention relates to a plasmid vector usable in genetic recombinant technology and to a method for expressing a gene by using the plasmid vector. The present invention also relates to a method for isolating a 10 desired gene by using such a plasmid vector. In addition, the present invention relates to a restriction enzyme and a gene thereof available as a genetic engineering reagent, in more detail to the AccIII restriction endonuclease and a DNA coding therefor.

15       BACKGROUND ART

In constructing an expression system for a desired gene by genetic recombinant technology, expression of the gene is controlled by bringing it under control of a promoter recognized by the RNA polymerase of the host used. In the case of a gene encoding a protein harmful to the host, however, plasmid construction itself is sometimes hampered by expression of the product of the gene due to the inability to stringently control the expression of the promoter used.

25       As an expression system resolving that problem, the

pET system (produced by Novagen) has been developed, which uses the RNA polymerase of the bacteriophage T7, which infects *Escherichia coli*, with *Escherichia coli* as a host [Journal of Molecular Biology, Vol. 189, pp. 113-130  
5 (1986); Gene, Vol. 56, pp. 125-135 (1987)]. The pET system is a system that allows T7 RNA polymerase, which has high promoter recognition specificity and high transcription activity, to be expressed in *Escherichia coli*, which T7 RNA polymerase transcribes a desired gene placed downstream of the T7 promoter on an expression vector and causes high expression of the gene. Because transcription of the desired gene occurs in the presence of T7 RNA polymerase, plasmid construction in the host is possible without expressing the desired gene, provided  
10 that the host does not produce the polymerase; plasmid construction itself is never hampered, as in cases where the expression system is constructed, while the desired gene is kept under control of a promoter recognized by the RNA polymerase of the host.

20 However, because the T7 RNA polymerase gene has been cloned onto the λ-phage vector and lysogenized into the expression host, there is no freedom of host choice; painstaking procedures are needed if the host is changed. In addition, because the expression of T7 RNA polymerase  
25 in the host is not stringently controlled, T7 RNA

polymerase is expressed even when the host is in a non-inductive condition, resulting in expression of the desired gene placed downstream of the T7 promoter on the expression vector even in a non-inductive condition. To suppress such expression of the desired gene in a non-inductive condition, T7 RNA polymerase activity is inhibited using T7 lysozyme, a T7 RNA polymerase inhibitor [Journal of Molecular Biology, Vol. 219, pp. 37-44 (1991)], or T7 RNA polymerase is prevented from getting access to the T7 promoter by placing a lactose operator downstream of the T7 promoter [Journal of Molecular Biology, Vol. 219, pp. 45-59 (1991)].

However, even these countermeasures are unsatisfactory in terms of effect against T7 RNA polymerase of high transcription activity so that the activity of T7 RNA polymerase in a non-inductive condition cannot be completely inhibited. For this reason, if the desired gene product is lethal to the host, it is impossible in some cases to prepare a transformant for expression of the gene, even when plasmid construction is possible. In other words, the pET system involves two problems to be resolved: one of the inability to freely change the host, and the other of inaccurate control of T7 RNA polymerase expression.

On the other hand, there is a bacteriophage having

characteristics similar to those of the bacteriophage T7,  
known as the bacteriophage SP6 [Science, Vol. 133, pp.  
2069-2070 (1961)], which infects *Salmonella typhimurium*.  
The RNA polymerase produced by the bacteriophage SP6, a  
5 single peptide having a molecular weight of about 100,000,  
is commonly used for in vitro RNA synthesis since it  
possesses high promoter recognition specificity and high  
transcription activity [Journal of Biological Chemistry,  
Vol. 257, pp. 5772-5778 (1982); Journal of Biological  
10 Chemistry, Vol. 257, pp. 5779-5788 (1982)]. In addition,  
the SP6 RNA polymerase gene has already been cloned and  
expressed in large amounts in *Escherichia coli* [Nucleic  
Acids Research, Vol. 15, pp. 2653-2664 (1987)].

Genes whose expression product acts lethally on hosts  
15 are exemplified by restriction endonuclease genes.  
Essentially, restriction endonucleases are utilized for  
self-defence by cleaving phages and other exogenous DNA  
entering the cells of microorganisms that produce the  
restriction endonucleases. On the other hand,  
20 microorganisms that produce restriction endonucleases  
mostly produce modification enzymes that recognize the  
same base sequences as those of the restriction  
endonucleases, to protect their own DNA against cleavage  
by the restriction endonucleases. Specifically, a  
25 modification enzyme modifies DNA by adding a methyl group

to one or more bases in the base sequence recognized thereby, to make it impossible for the restriction endonuclease that recognizes the same sequence as that of the modification enzyme to bind thereto or to cleave the DNA. This mechanism is called restriction modification system, and the pair of genes of the restriction endonuclease and modification enzyme that constitute the restriction modification system called restriction modification system gene. Therefore, when the restriction endonuclease gene is expressed in a microorganism lacking a modification enzyme gene from the restriction modification system gene, the microorganism's DNA is cleaved, resulting in cell death. In fact, there are two modification enzyme genes in the MboI restriction modification system gene; it has been reported that cloning of restriction endonuclease genes is impossible due to incomplete modification of the host DNA in the case of incomplete methylation in the co-presence of either modification enzyme gene alone [Nucleic Acids Research, Vol. 21, pp. 2309-2313 (1993)].

Also, it has been demonstrated that if a restriction modification system gene is lost from a cell retaining the restriction modification system gene, a lack of modification activity in the cell results in incomplete methylation of genomic DNA, which in turn causes lethal

cleavage of its own genomic DNA by a very small amount of restriction endonuclease remaining therein [Science, Vol. 267, pp. 897-899 (1995)]. In summary, in the absence of modification enzymes that constitute a restriction modification system, restriction endonucleases behave as proteins very harmful to cells; separate cloning and expression of their genes have been impossible by prior art technologies.

Concerning restriction endonucleases, restriction endonucleases can be classified by their enzymatic properties into three types: I, II and III. Type II restriction endonucleases, in particular, each of which recognizes a particular DNA base sequence and cleaves it at a particular site in or near the sequence, are extensively used in the field of genetic engineering, and restriction endonucleases of this type with various specificities have been isolated from a variety of microorganisms [Nucleic Acids Research, Vol. 24, pp. 223-235 (1996)]. In the present specification, a type II restriction endonuclease is hereinafter referred to as "restriction endonuclease". It should be noted, however, that some microorganisms produce only small amounts of restriction endonuclease, and others produce a plurality of restriction endonucleases. For example, the restriction endonuclease AccIII is produced by

Acinetobacter calcoaceticus (hereinafter referred to as Acc bacterium), which has been deposited under accession number FERM BP-935 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry [address: 1-3, Higashi 1-chome, Yatabemachi, Tsukuba-gun, Ibaraki, 305, Japan] since November 9, 1985 (date of original deposition), but the amount of the enzyme produced is small and this microorganism also produces the restriction endonucleases AccI and AccII simultaneously.

Therefore, advanced production technology is needed to provide the restriction endonuclease AccIII as a reagent of high purity and low cost using this microorganism. In providing a restriction endonuclease as a reagent of high purity and low cost, it is effective to isolate the desired restriction endonuclease gene and selectively produce the desired restriction endonuclease in large amounts by genetic engineering technology. To accomplish this purpose, some methods of isolating restriction endonuclease genes have been reported.

First, there may be mentioned the "shotgun" method, wherein the genomic DNA of a microorganism that produces a restriction endonuclease is cleaved using the appropriate restriction endonuclease, the resulting fragment is inserted into an appropriate plasmid vector, and a clone

expressing the restriction endonuclease gene is selected. Screening methods for desired clones are exemplified by a method wherein a restriction modification system gene is isolated with resistance to phage infection as an index, 5 on the basis of the self-defence function acquired by the host upon introduction of the restriction modification system gene thereinto [PstI: Proceedings of the National Academy of Science of the USA, Vol. 78, pp. 1503-1507 (1981)]. This method, however, necessitates that the size 10 of the restriction modification system gene falls within a range allowing its isolation, and that the expression of the restriction modification system gene isolated exhibits sufficient phage resistance to allow the selective survival of the host. On the other hand, as a general 15 feature of restriction modification system genes, there may be mentioned the close location on the genome of restriction endonuclease genes and modification enzyme genes; in fact, this has been confirmed in many restriction modification system genes that have so far 20 been obtained [Nucleic Acids Research, Vol. 19, pp. 2539-2566 (1991)]. Accordingly, there is a method wherein a restriction modification system gene is screened for with the expression of a modification enzyme gene as an index on the basis of the above-described feature 25 [Japanese Patent Laid-Open No. 63-87982; Nucleic Acids

Research, Vol. 19, pp. 1831-1835 (1991)]. When the restriction endonuclease gene is not close to the modification enzyme gene, however, this method fails to yield the restriction endonuclease gene.

5 Furthermore, the above-described "shotgun" method poses a fundamental problem associated with a difference in transcription-translation mechanism between the genomic DNA source organism and the host. For example, in the case of insufficient gene expression due to the failure of  
10 the promoter and ribosome binding site accompanying the restriction modification system gene to function well in the host, much labor is needed to select transformants containing the desired gene, even if obtained. To avoid this drawback, there is a method wherein the amino acid  
15 sequence of the restriction endonuclease protein is analyzed, the restriction endonuclease gene is obtained from the genomic DNA of a microorganism that produces the restriction endonuclease by PCR-based DNA amplification on the basis of the sequence data obtained, and wherein a  
20 known protein expression system is utilized [Japanese Patent Laid-Open No. 6-277070]. Because the presence of a restriction endonuclease is lethal to the host in conventional protein expression systems, there is a need to protect the host by, for example, allowing a  
25 modification enzyme that constitutes a restriction

modification system together with the enzyme to be co-present.

Although all the above-described methods of the isolation of restriction endonuclease genes necessitate 5 the simultaneous isolation of the restriction endonuclease gene and a modification enzyme gene that constitutes a restriction modification system gene together with the gene, another method of isolating the restriction endonuclease gene alone has been reported [Nucleic Acids 10 Research, Vol. 22, pp. 2399-2403 (1994)]. In that method, however, it is intended to isolate a gene encoding a restriction endonuclease for which optimal temperature for 15 enzyme activity is around 70°C; the co-presence of a modification enzyme gene is necessary when the gene to be isolated encodes a restriction endonuclease showing high specific activity near host culturing temperature.

Exceptionally, there are restriction endonucleases that do not show cleavage activity unless a particular nucleic acid base in their DNA recognition sequence has 20 not been modified by methylation, like the restriction endonuclease DpnI. Genes for restriction endonucleases possessing this property are thought to be exceptional in that they can be isolated even in the absence of another particular gene by selecting the appropriate host 25 organism. In fact, the mrr gene has been isolated, which

encodes the Mrr protein, which is not a type II  
restriction endonuclease but which recognizes a particular  
DNA base sequence containing a methylated nucleic acid  
base and exhibits DNA cleavage activity [Journal of  
5 Bacteriology, Vol. 173, pp. 5207-5219 (1991)].

As stated above, isolation of a restriction  
endonuclease gene by the prior art necessitates the  
simultaneous expression of the gene and a modification  
enzyme gene that constitutes a restriction modification  
10 system gene together with the gene, except for special  
cases.

DISCLOSURE OF THE INVENTION

Therefore, a first object of the present invention  
15 provides a plasmid vector capable of isolating such a gene  
that an isolation thereof or a construction of an  
expression system thereof has been difficult in the prior  
arts because the gene product is lethal or harmful to a  
host, and capable of introducing into a host to express  
20 the protein efficiently. A second object of the present  
invention provides a method for expressing a desired gene  
by using this plasmid vector. A third object of the  
present invention provides a method for isolating a  
desired gene by using this plasmid vector, especially for  
25 isolating a restriction endonuclease gene without co-

existence of a modification enzyme gene constituting a restriction modification system. A fourth object of the present invention provides a polypeptide possessing an activity of an AccIII restriction endonuclease. In  
5 addition, a fifth object of the present invention provides a DNA which encodes a polypeptide possessing an activity of an AccIII restriction endonuclease.

First, to resolve the problems in the pET system, the present inventors have constructed an expression system  
10 using SP6 RNA polymerase as a new accurate expression control expression system, and assessed the system.

Because the expression system is constructed using a system plasmid inserted the SP6 RNA polymerase gene into the minIF plasmid, expression systems for the desired gene  
15 can be constructed in various strains of *Escherichia coli* by simultaneously introducing an expression vector harboring the desired gene cloned downstream of the SP6 promoter and this system plasmid into the host. In addition, using the lac promoter and antisense technology,  
20 the present inventors made it possible to exactly control the expression of the SP6 RNA polymerase gene on the system plasmid. Assessing this expression system using the  $\beta$ -galactosidase gene as a reporter gene demonstrated that the expression of the SP6 RNA polymerase gene is  
25 accurately controlled in this system, that there is almost

no expression of SP6 RNA polymerase in a non-inductive condition, and that induction is followed by the expression of a sufficient amount of SP6 RNA polymerase to efficiently express the desired gene and subsequent 5 expression of the desired gene at a high level.

It was also shown, however, that when the host used is *Escherichia coli*, the desired gene downstream of the SP6 promoter is expressed in very small amounts even in a non-inductive condition because the SP6 promoter is very 10 weakly but actually recognized by *Escherichia coli* RNA polymerase. It was thus proven that when the gene product acts very harmfully and lethally to *Escherichia coli*, expression system construction is impossible so that the object of the present invention cannot be accomplished 15 well.

With these findings in mind, the present inventors have made further extensive investigation and unexpectedly found that (i) expression of the desired gene in a non-inductive condition can be suppressed to undetectable 20 levels, and that (ii) expression induction increases the copy number of the plasmid containing the desired gene and causes RNA polymerase expression, resulting in the transcription and translation of the desired gene placed downstream of a promoter recognized by the RNA polymerase, 25 by using a new system for controlling the expression of

the desired gene by means of a combination of two control methods, i.e., control of the copy number of the gene, and transcription control via the promoter. In other words,  
the plasmid vector of the present invention is a plasmid  
5 vector having unique features to resolve the above problems in the field of genetic engineering. The present inventors made further investigation based on this finding, and developed a method for expressing the desired gene using the vector.

10 The present inventors also have developed a method for isolating a gene whose expression product acts lethally on the host, using the above-described plasmid vector, more specifically a method of isolating a restriction endonuclease gene without the drawbacks of the  
15 prior art.

The present inventors also found it possible to isolate DNA encoding the AccIII restriction endonuclease, which had not been obtained so far, without the co-presence of an AccIII modification enzyme, and express  
20 the AccIII restriction endonuclease in large amounts, using the above-described method.

(1) The gist of the present invention is concerned with:  
A plasmid vector characterized by comprising a promoter sequence to control an expression of a desired gene, the  
25 promoter sequence being recognized by an RNA polymerase

not inherent to a host, and a replication origin for increasing a copy number by induction with an exogenous factor;

- (2) The plasmid vector described in item (1) above,  
5 wherein the promoter sequence is recognized by RNA polymerases derived from bacteriophages;
- (3) The plasmid vector described in item (2) above,  
wherein the promoter sequence is recognized by an RNA  
polymerase derived from SP6 phage;
- 10 (4) The plasmid vector described in item (3) above,  
wherein the promoter sequence contains the base sequence  
of SEQ ID NO:30 in the Sequence Listing;
- (5) The plasmid vector described in any one of items (1)  
to (4) above, wherein the replication origin is under  
15 control of a promoter;
- (6) The plasmid vector described in any one of items (1)  
to (5) above, wherein the replication origin is under  
control of the lac promoter;
- (7) The plasmid vector described in any one of items (1)  
20 to (6) above, comprising a drug resistance gene as a  
selection marker;
- (8) The plasmid vector described in item (7) above, which  
is selected from pACE601, pACE611, pACE701 and pACE702;
- (9) A plasmid vector in which a desired gene to be  
25 expressed is incorporated into the plasmid vector

described in any one of items (1) to (8) above;

- (10) A method for expressing a desired gene, characterized by introducing into a host a plasmid vector in which the  
5 desired gene is incorporated into the plasmid vector described in any one of items (1) to (8) above, and an RNA polymerase gene which recognizes a promoter sequence in the plasmid vector, and inducing an increase in a copy number of the plasmid vector and an expression of the RNA  
10 polymerase by using an exogenous factor to transcribe and translate the desired gene;
- (11) The method for expressing a desired gene described in item (10) above, characterized in that the increase in the copy number of the plasmid vector and the expression of  
15 the RNA polymerase are induced by respective exogenous factors;
- (12) The method for expressing a desired gene described in item (10) above, characterized in that the increase in the copy number of the plasmid vector and the expression of  
20 the RNA polymerase are induced by a same exogenous factor;
- (13) The method for expressing a desired gene described in any one of items (10) to (12) above, wherein the exogenous factor which induces the increase in the copy number of the plasmid vector, is one or more selected from the group  
25 consisting of an addition of isopropyl- $\beta$ -D-thiogalactoside

(IPTG), an addition of lactose, an addition of galactose, an addition of arabinose, a reduction of a tryptophane concentration and an adjustment of a transformant cultivation temperature;

- 5       (14) The method for expressing the desired gene described in any one of items (10) to (12) above, wherein the exogenous factor which induces the expression of the RNA polymerase, is one or more selected from the group consisting of an addition of isopropyl- $\beta$ -D-thiogalactoside
- 10      (10) (IPTG), an addition of lactose, an addition of galactose, an addition of arabinose, a reduction of a tryptophane concentration and an adjustment of a transformant cultivation temperature;
- 15      (15) The method for expressing a desired gene described in item (10) above, characterized in that the RNA polymerase gene is introduced into the host by the other plasmid vector or a phage vector;
- 20      (16) The method for expressing a desired gene described in item (10) above, characterized in that the RNA polymerase gene is incorporated into a chromosome of the host;
- 25      (17) The method for expressing a desired gene described in item (15) or item (16) above, characterized in that the RNA polymerase gene is derived from SP6 phage;
- (18) The method for expressing a desired gene described in any one of items (10) to (17) above, wherein the desired

gene encodes a protein lethal or harmful to the host;

(19) The method for expressing a desired gene described in any one of items (10) to (18) above, characterized in that *Escherichia coli* is used as the host;

5 (20) A method for isolating a desired gene, characterized in that the plasmid vector described in any one of items (1) to (8) above is employed in the method for isolating the desired gene;

10 (21) The method for isolating a desired gene described in item (20) above, wherein the desired gene encodes a protein lethal or harmful to a host;

15 (22) The method for isolating a desired gene described in item (21) above, wherein the gene encoding a protein lethal or harmful to the host is a restriction endonuclease gene;

(23) A polypeptide containing the entire or a portion of the amino acid sequence shown by SEQ ID NO:1 in the Sequence Listing, and possessing an activity of AccIII restriction endonuclease;

20 (24) A polypeptide having an amino acid sequence resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:1 in the Sequence Listing or a portion thereof, and possessing an activity of AccIII restriction endonuclease;

25

- (25) A DNA encoding a polypeptide which contains the entire or a portion of the amino acid sequence shown by SEQ ID NO:1 in the Sequence Listing, and possesses an activity of AccIII restriction endonuclease;
- 5 (26) A DNA containing the entire or a portion of the DNA shown by SEQ ID NO:2 in the Sequence Listing wherein an expression product of the DNA possesses an activity of AccIII restriction endonuclease;
- 10 (27) A DNA encoding a polypeptide resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:1 in the Sequence Listing or a portion thereof, and possessing an activity of AccIII restriction endonuclease; and
- 15 (28) A DNA capable of hybridizing to the DNA described in any one of items (25) to (27) above, and encoding a polypeptide possessing an activity of AccIII restriction endonuclease.

20 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the constitution of the model expression plasmids pMSP6L and pMSP6F.

Figure 2 shows the constitution of the model expression plasmid pMSP60.

25 Figures 3 (A), (B) show the procedure of the

construction of the runaway plasmid pHS2870.

Figure 4 shows the constitution of the runaway plasmid pHS2870.

Figures 5 (A), (B) show the procedure of the  
5 construction of the plasmid pCRS04.

Figure 6 shows the constitution of the plasmid pCRS04.

Figure 7 shows the constitution of the plasmid pCRA19.

10 Figures 8 (A), (B), (C), (D), (E) show the procedure of the construction of the system plasmid pFSP6.

Figures 9 (A), (B) show the procedure of the construction of the plasmid pACE601.

15 Figure 10 shows the constitution of the plasmid pACE611.

Figures 11 (A), (B) show the procedure of the construction of the plasmid pACE611.

Figures 12 (A), (B) show the procedure of the construction of the plasmid pCRS70.

20 Figures 13 (A), (B) show the procedure of the construction of the plasmids pACE701 and pACE702.

Figure 14 shows the expression of the activity of Nsp 7524 III restriction endonuclease depending on the induction or non-induction of IPTG, by agarose gel 25 electrophoresis of the degradation reaction solution of  $\lambda$ -

DNA after introducing pFSP6 and pACE601 or pFSP6 and pACE601-NspIII into *Escherichia coli* HB101. In this figure, M indicates the  $\lambda$ -EcoT141 size marker; 1 indicates AvaI digested  $\lambda$ -DNA; 2 indicates HB101/pFSP6/pACE601 after induction; 3 indicates HB101/pFSP6/pACE601 before induction; 4 indicates HB101/pFSP6/pACE601-NspIII after induction; and 5 indicates HB101/pFSP6/pACE601-NspIII before induction.

Figure 15 shows a result of an agarose gel electrophoresis in the case of 2 and 3 hour-elongation of the degradation reaction of  $\lambda$ -DNA depending on the induction or non-induction of IPTG after introducing pFSP6 and pACE601 or pFSP6 and pACE601-NspIII into *Escherichia coli* HB101. In this figure, M indicates the  $\lambda$ -EcoT141 size marker; 1 indicates AvaI digested  $\lambda$ -DNA; 2 indicates HB101/pFSP6/pACE601 after 1 hour (induction); 3 indicates HB101/pFSP6/pACE601 after 2 hours (induction); 4 indicates HB101/pFSP6/pACE601 after 3 hours (induction); 5 indicates HB101/pFSP6/pACE601 after 1 hour (non-induction); 6 indicates HB101/pFSP6/pACE601 after 2 hours (non-induction); 7 indicates HB101/pFSP6/pACE601 after 3 hours (non-induction); 8 indicates HB101/pFSP6/pACE601-NspIII after 1 hour (induction); 9 indicates HB101/pFSP6/pACE601-NspIII after 2 hours (induction); 10 indicates HB101/pFSP6/pACE601-NspIII after 3 hours (induction); 11

indicates HB101/pFSP6/pACE601-NspIII after 1 hour (non-induction); 12 indicates HB101/pFSP6/pACE601-NspIII after 2 hours (non-induction); and 13 indicates HB101/pFSP6/pACE601-NspIII after 3 hours (non-induction).

5       Figure 16 shows the construction of the AccIII restriction modification system gene.

BEST MODE FOR CARRYING OUT THE INVENTION

The first aspect of the present invention relates to  
10      a plasmid vector. More specifically, it relates to a plasmid vector characterized in that the plasmid vector comprises a promoter sequence, which is recognized by an RNA polymerase not inherent to the host, to control the expression of the desired gene, and a replication origin  
15      for increasing the copy number by induction with an exogenous factor. The promoter sequence contained in the plasmid vector, which sequence controls the expression of the desired gene, may be any promoter sequence; promoter sequences recognized by particular RNA polymerases, e.g.,  
20      those recognized by RNA polymerases derived from the T7, T3, SP6 and other bacteriophages, can be used, with preference given to those recognized by the RNA polymerase derived from the SP6 phage. The base sequence of the minimum region of the promoter recognized by the RNA  
25      polymerase derived from the SP6 phage, i.e., the SP6

promoter, is shown by SEQ ID NO:30 in the Sequence Listing. Furthermore, the plasmid vector may have a drug resistance gene used as a selection marker.

Exogenous factors that control the copy number of the plasmid vector of the present invention, i.e., the function of the replication origin of the plasmid vector, include, but are not limited to, for example, the addition of various chemicals, such as lactose or structurally related substances like isopropyl- $\beta$ -D-thiogalactoside (IPTG), galactose or structurally related substances, and arabinose or structurally related substances; reduction of tryptophane concentration; and adjustment of transformant cultivation temperature. By bringing the replication origin under control of a promoter that can be controlled by these factors, copy number control becomes possible. Promoters usable for this purpose include, but are not limited to, the lac, trp, tac, gal, ara and  $P_L$  promoters etc. when the host used is *Escherichia coli*, as long as the above-described purpose is accomplished. These promoters can also be used to induce the expression of an RNA polymerase gene.

Examples of the replication origin of the plasmid vector of the present invention include, but are not limited to, plasmid vectors that serve as replication origins of runaway plasmids under control of the lac

promoter. In this case, copy number control is achieved by the addition of lactose and structurally related substances, most preferably by the addition of isopropyl- $\beta$ -D-thiogalactoside (IPTG).

5       The second aspect of the present invention relates to a method for expressing a desired gene to be expressed, characterized by introducing a plasmid vector prepared by incorporating the desired gene into the plasmid vector of the present invention, and an RNA polymerase gene that  
10      recognizes the promoter sequence on the plasmid vector, into a host, and inducing an increase in the copy number of the plasmid vector and the expression of the RNA polymerase with an exogenous factor to transcribe and translate the desired gene. RNA polymerase genes that  
15      recognize the promoter sequence on the plasmid vector include, but are not limited to, for example, the RNA polymerase genes derived from the above-mentioned bacteriophages, with preference given to the RNA polymerase gene derived from the SP6 phage. Exogenous  
20      factors that induce the expression of such RNA polymerase or an increase in the copy number of the plasmid vector include, but are not limited to, for example, the addition of various chemicals, such as lactose or structurally related substances like isopropyl- $\beta$ -D-thiogalactoside  
25      (IPTG), galactose or structurally related substances, and

arabinose or structurally related substances; the reduction of tryptophane concentration; and the adjustment of transformant cultivation temperature.

Induction of an increase in the copy number of plasmid vector and that of expression of RNA polymerase may be achieved by the action of respective exogenous factors or a same exogenous factor. Also, in this case, the gene encoding RNA polymerase may be incorporated onto a chromosome of the host, or introduced into the host by a plasmid vector other than the plasmid vector of the present invention or a phage vector. In the latter case, the host can readily be changed according to the purpose. In that case, introduction of the vector having an RNA polymerase gene into the host may precede or follow the introduction of the plasmid vector of the present invention.

Although the desired gene in the present invention is not subject to limitation, it is meaningful when it encodes a protein lethal or harmful to the host. Such proteins include, for example, the above-mentioned restriction endonucleases, other nucleases, nucleic acid-binding proteins, and proteases.

The present invention provides a system that controls the expression of a desired gene by a combination of two control methods, i.e., control of the copy number of the

gene, and control of transcription via a promoter, as described above. Control of the expression of the desired gene cloned onto a plasmid in the present invention, unlike conventional control, is very stringent so that its expression can be suppressed to an undetectable level in a non-inductive condition. When the copy number of the plasmid, i.e., the copy number of the desired gene, is increased, with concurrent expression of RNA polymerase, by expression induction, the desired gene is transcribed and translated under control of the promoter by the action of the RNA polymerase.

It is therefore possible to use the plasmid vector of the present invention to prepare a transformant containing a gene harmful or lethal to the host and express the gene, a task difficult to achieve by the prior art.

For example, when cloning of a gene encoding the Nsp7524 III restriction endonuclease was attempted using the plasmid vector of the present invention, the gene was successfully retained in *Escherichia coli* without co-presence of the corresponding modification enzyme gene. It was also possible to express the Nsp7524 III restriction endonuclease in the cells of the resulting transformant by introducing a system plasmid containing an RNA polymerase gene into the transformant to induce the expression of the Nsp7524 III restriction endonuclease.

Also, using the plasmid vector of the present invention as a cloning vector for preparation of a gene library, it is possible to isolate a gene that cannot be isolated by a conventional method, and confirm the 5 activity of its expression product in a single host. The use of the plasmid vector of the present invention is of course not limited to the cloning of a gene encoding a product harmful to the host, and can be used as a general-purpose plasmid vector.

10 An RNA polymerase gene relating to the present invention can be introduced into a host using, for example, the plasmid pFSP6 and the phage M13sp6. Details of construction of the plasmid pFSP6 are shown in Reference Example (2). *Escherichia coli* HB101 as 15 transformed with the plasmid, designated *Escherichia coli* HB101/pFSP6, has been deposited under accession number FERM BP-5742 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry 20 [address: 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, 305, Japan] since December 22, 1995 (date of original deposition). A method for the construction of the phage M13sp6 is described in Example 2 (6).

The present invention is hereinafter described in 25 more detail with reference to a case using the plasmid

pFSP6.

First described are the results of an experiment conducted using the constructed multicopy model expression plasmids pMSP6L, pMSP6F (Figure 1) and pMSP6O (Figure 2), all having a promoter sequence recognized by SP6 RNA polymerase and the  $\beta$ -galactosidase gene as a reporter gene, and all allowing the expression of the desired gene using the above-described system plasmid pFSP6.

Details of construction of these model expression plasmids are shown in Reference Example (3). These plasmids incorporate the minimum region of the SP6 promoter (pMSP6L), a inherent SP6 promoter region (pMSP6F), or both the minimum region of the SP6 promoter and the lac operator region (pMSP6O), respectively, as an SP6 plasmid sequence.

When the plasmid pMSP6L or pMSP6F alone was introduced into the *Escherichia coli* strains shown in Table 1,  $\beta$ -galactosidase activity was noted, despite the fact that the host *Escherichia coli* did not express SP6 RNA polymerase, the amounts being higher than that obtained with the plasmid pMS434, which does not have the SP6 promotor, as shown in Table 2. In short, it can be conjectured that the SP6 promoter is recognized by the RNA polymerase derived from *Escherichia coli*, and that the  $\beta$ -galactosidase gene downstream thereof is transcribed and

translated. Also, when *Escherichia coli* MRi80, a strain that has a mutation in the *pcnB* gene and wherein the copy number of a plasmid having a replication origin derived from *Colel*, like the above-described model of expression plasmids, is reduced by 1/6 to 1/13 of that of the parent strain MRi7, was used as a host, the  $\beta$ -galactosidase activity was also decreased to 1/7 to 1/14 of that of MRi7. This suggests a correlation between the  $\beta$ -galactosidase gene expression level and the copy number of the plasmid introduced.

Table 1

Strain	Genotype	Origin / Reference
MC4100	F-, araD139, $\Delta$ (argF-lac)U169, thiA, rpsL150, relA1, f1bB5301, deoC1, ptsF25, rbsR	Casadaban et al. J. Mol. Biol. 104(1976) 541-555.
MRi7	MC4100 $\Delta$ rbs-7	Lopilato et al. J. Bacteriol. 158(1984) 665-673
MRi80	MRi7 <i>pcnB</i> 80	Lopilato et al. Mol. Gen. Genet. 205(1986) 285-290.

Table 2

Strain	$\beta$ - Galactosidase Activity		
	Plasmid		
	pMSP6F	pMSP6L	pMS434
MC4100	202	242	30
MRi7	234	287	23
MRi80	17	27	4

Next, when the same model of expression plasmids were  
5 introduced into *Escherichia coli* together with the  
above-described system plasmid pFSP6 and  $\beta$ -galactosidase  
activity was determined under conditions such that the  
expression of the SP6 RNA polymerase gene was not induced,  
the  $\beta$ -galactosidase activity was at most as high as that  
10 obtained with *Escherichia coli* not containing the system  
plasmid, as shown in Table 3. This demonstrates that the  
expression of the SP6 RNA polymerase gene on the plasmid  
pFSP6 is almost completely suppressed in a non-inductive  
condition. On the other hand, when the expression of the  
15 SP6 RNA polymerase gene was induced by the addition of  
isopropyl- $\beta$ -D-thiogalactoside (IPTG),  $\beta$ -galactosidase  
activity was increased by 18 to 32 times, demonstrating  
that the  $\beta$ -galactosidase gene can be expressed via the SP6  
promoter. When the host used was *Escherichia coli* MRi80,  
20 in particular,  $\beta$ -galactosidase activity was increased to a  
level 25 to 32 times that obtained in a non-inductive  
condition, 4 hours after induction, though the activity in  
a non-inductive condition remained at a very low level.

Table 3

Strain	$\beta$ -Galactosidase Activity (Non-Induction/Induction)					
	pMSP6F		pMSP6L		pMS434	
	Non-Induction	Induction	Non-Induction	Induction	Non-Induction	Induction
MC4100(pFSP6)	221	4875	234	5256	25	19
MRi7(pFSP6)	220	3958	242	4243	25	21
MRi80(pFSP6)	17	425	30	970	3	ND

10

In the plasmid pMSP6O, which carries the lac operator sequence downstream of the SP6 promoter, the  $\beta$ -galactosidase activity under non-inductive conditions is lower than that in other model expression plasmids;

15 however, its expression cannot be suppressed completely. These results suggest that the expression of the desired gene is difficult to completely suppress in an expression system using a multicopy plasmid, and that controlling the copy number of the plasmid containing the desired gene is 20 more effective in resolving this problem than controlling the expression of the RNA polymerase gene on the system plasmid.

The present inventors thus made further investigation based on these results to explore a new expression vector, 25 and developed the plasmid vector of the present invention.

Specifically, the present inventors constructed a runaway plasmid vector capable of reducing the expression level in a non-inductive condition to ensure sufficient expression after induction. First, in consideration of 5 the fact that SP6 RNA polymerase expression in a system plasmid is induced by IPTG, a plasmid having a runaway replication origin allowing an increase in copy number by IPTG induction was constructed in accordance with the procedures shown in Figure 3. First, the plasmid 10 pUC106AdPO is constructed by removing the PvuII fragment containing the lac promoter and the operator region from the plasmid pUC106A, which is obtained by introducing the 106 NdeI DNA fragment prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:3 and SEQ ID 15 NO:4 in the Sequence Listing into the NdeI site of the plasmid vector pUC19 (produced by Takara Shuzo).

Next, the plasmid pHs2870 can be obtained by inserting the RNA1870 fragment obtained by PCR using the RNAlIA primer (base sequence shown by SEQ ID NO:5 in the 20 Sequence Listing) arranged near the RNAlI region (replication origin of the plasmid) and the 1870 primer (base sequence shown by SEQ ID NO:6 in the Sequence Listing) arranged at a terminus of the 106 NdeI sequence with the plasmid as a template, into the XbaI site of the 25 plasmid pSTV28 (produced by Takara Shuzo). The plasmid

pHS2870 thus constructed is shown in Figure 4. Generally, the plasmid is present at a copy number of about 30 copies per *Escherichia coli* cell; however, this copy number is increased to several hundreds by the addition of IPTG.

5       The AccI-NspI fragment containing the P15A replication origin derived from the plasmid pSTV28 is then removed from the plasmid (plasmid pCRS01), followed by further removal of the EcoRI-XbaI fragment containing an unnecessary restriction endonuclease site (plasmid 10 pCRS02), after which a DNA fragment containing the lactose repressor (*lacIq*) gene derived from the plasmid pMJR1560 [Gene, Vol. 51, pp. 225-267 (1987)] is introduced to yield the plasmid pCRS04. A flow diagram of the construction of the plasmid pCRS04 from the plasmid pHS2870 is shown in 15 Figure 5. The plasmid pCRS04 thus constructed is shown in Figure 6. The plasmid is a runaway plasmid induced by IPTG; generally, the plasmid is present at a copy number of 1 to 2 copies per *Escherichia coli* cell; however, this copy number is increased to several hundreds by the 20 addition of IPTG.

To control the expression of the desired gene inserted into the plasmid, the  $P_{SP6}$ -O<sub>lac</sub> EX linker, a double-stranded oligonucleotide containing the SP6 promoter sequence and lac operator sequence, may be 25 introduced into the NheI site of the plasmid to construct

the plasmid pACE601. The  $P_{SP6}-O_{lac}$  EX linker is prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:7 and SEQ ID NO:8 in the Sequence Listing. The plasmid pACE601 is a runaway plasmid vector having the 5 chloramphenicol resistance gene as a selection marker.

Also, by introducing the BspHI fragment containing the  $\beta$ -lactamase gene derived from the plasmid vector pUC118 (produced by Takara Shuzo) into the NheI site of the above-described plasmid pCRS04 (plasmid pCRS70), 10 subsequently removing the NcoI-BsaAI fragment containing the chloramphenicol resistance gene, and replacing it with the above-described  $P_{SP6}-O_{lac}$  EX linker, the plasmids pACE701 and pACE702 can be constructed, which plasmids have the linker inserted in mutually opposite directions. These 15 plasmids are runaway plasmid vectors having the ampicillin resistance gene as a selection marker.

The potential of the thus-obtained plasmids pACE601, pACE701 and pACE702 used for control of the expression of the desired gene can be determined using the 20  $\beta$ -galactosidase gene as a reporter gene, as described above. It is possible to construct model expression plasmids by introducing a DNA fragment containing the  $\beta$ -galactosidase gene as amplified by PCR using the primers trpA-N-NcoI and lacZ-C-NcoI with the plasmid pMS434 [Gene, 25 Vol. 57, pp. 89-99 (1987)] as a template, into the NcoI

site downstream of the SP6 promoter in each of the above-described three plasmids. The base sequences of the primers trpA-N-NcoI and lacZ-C-NcoI are shown by SEQ ID NO:9 and SEQ ID NO:10 in the Sequence Listing,  
5 respectively. The plasmids thus obtained are designated pACE601Z, pACE701Z and pACE702Z, respectively. In *Escherichia coli* transformed with these model expression plasmids, absolutely no  $\beta$ -galactosidase activity is detected, demonstrating very exact control of the  
10 expression of the  $\beta$ -galactosidase gene.

Using the Nsp7524 III restriction endonuclease gene, it is possible to confirm that a gene whose expression product acts lethally on the host can be isolated and expressed using the plasmid of the present invention. The  
15 plasmid pBRN3 contains the Nsp7524 III restriction modification system gene. *Escherichia coli* MC1061 as transformed with the plasmid, designated *Escherichia coli* MC1061/pBRN3, has been deposited under accession number FERM BP-5741 at the National Institute of Bioscience and  
Human-Technology, Agency of Industrial Science and  
Technology, Ministry of International Trade and Industry  
[address: 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken,  
305, Japan] since September 28, 1995 (date of original  
deposition). By a PCR-based DNA amplification reaction  
20 using a pair of the primers L-ORF and NspR-ORF3 with the  
25

plasmid pBRN3 prepared from the transformant as a template, a DNA fragment containing the Nsp7524 III restriction endonuclease gene alone can be obtained. The base sequences of the primers L-ORF and NspR-ORF3 are 5 shown by SEQ ID NO:11 and SEQ ID NO:12 in the Sequence Listing, respectively. By introducing into *Escherichia coli* HB101 the plasmid pACE601-NspIII, which is obtained by inserting the resulting DNA fragment into the NcoI site downstream of the SP6 promoter of the above-described 10 plasmid pACE601, the transformant *Escherichia coli* HB101/pACE601-NspIII can be obtained. The transformant allows the host to stably retain the Nsp7524 III restriction endonuclease gene despite the absence of the Nsp7524 III modification enzyme gene.

15 Furthermore, the transformant *Escherichia coli* HB101/pFSP6/pACE601-NspIII can be prepared by introducing the above-described system plasmid pFSP6 into the transformant. By culturing the transformant and inducing expression by the addition of IPTG at the appropriate 20 time, the possibility of production of the Nsp7524 III restriction endonuclease in the culture can be confirmed.

The third aspect of the present invention provides a method for isolating a desired gene characterized by using the above-described plasmid vector. The isolation method 25 of the present invention is suitably applied to the

isolation of a gene whose expression product is lethal or harmful against the host, especially a restriction endonuclease gene. By using the present method, it is possible to isolate a restriction endonuclease gene that

5 has not been isolated so far, e.g., the AccIII restriction endonuclease gene, even in the absence of the AccIII modification enzyme gene. Isolation of a restriction endonuclease gene can be achieved not only by the "shotgun method", wherein the genomic DNA of a microorganism that

10 produces the desired enzyme is cleaved with the appropriate restriction endonuclease, and the resulting DNA fragment is inserted directly into, for example, the plasmid pACE611, but also by the use of a cassette library of the genomic DNA of a microorganism that produces the

15 restriction endonuclease. By obtaining a gene by DNA amplification with the cassette library as a template, it is possible to express a gene prepared from a microorganism whose transcription mechanism differs from that of the host. The method of isolating a restriction endonuclease gene using a cassette library is hereinafter

20 described with reference to an example involving the AccIII restriction endonuclease.

A cassette library of the genomic DNA of a microorganism that produces the AccIII restriction endonuclease, i.e., an Acc bacterium, can be prepared as

follows: Genomic DNA is extracted from the cell culture of an Acc bacterium, and digested with the appropriate restriction endonuclease, after which the resulting DNA fragment is ligated to a cassette having a protruding end 5 complementary to the fragment. Several similar cassette libraries are prepared using different restriction endonucleases for genomic DNA cleavage. These libraries are generically referred to as an Acc genomic cassette library. Next, the desired restriction endonuclease 10 protein is purified from the Acc bacterium, its amino acid sequence is partially determined, and a primer is synthesized on the basis of the sequence. Using this primer and cassette primer, a PCR-based DNA amplification reaction is carried out with each cassette library as a 15 template, to obtain a DNA fragment containing an AccIII restriction endonuclease gene fragment. The base sequence of the DNA fragment obtained is determined by, for example, direct sequencing of the PCR product, to determine the full-length base sequence of the AccIII 20 restriction endonuclease gene.

By designing a primer capable of amplifying the full-length sequence of the AccIII restriction endonuclease gene from the base sequence, and carrying out a PCR-based DNA amplification reaction using this primer 25 with the genomic DNA of the Acc bacterium as a template, a

DNA fragment containing the full-length sequence of the AccIII restriction endonuclease gene is obtained. The DNA fragment obtained is inserted downstream of the SP6 promoter of the plasmid pACE611 so that the codon frames are adjusted, and the resulting recombinant plasmid is introduced into, for example, *Escherichia coli* JM109, to yield transformants. Transformants containing the AccIII restriction endonuclease gene in an expressible condition can be selected by culturing each transformant, inducing gene expression by the gene expression method of the present invention, and determining the AccIII restriction endonuclease activity in each culture obtained, as well as by drawing the restriction endonuclease map of the plasmid DNA harbored by each transformant.

The AccIII restriction endonuclease gene thus obtained was actually inserted into the plasmid pACE611, and the resulting plasmid was designated pCRA19. The restriction endonuclease map of this plasmid is shown in Figure 7, wherein the bold solid line indicates the DNA fragment containing the AccIII restriction endonuclease gene. *Escherichia coli* JM109 as incorporating the plasmid pCRA19, designated *Escherichia coli* JM109/pCRA19, has been deposited under accession number FERM BP-5743 at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of

International Trade and Industry [address: 1-3, Higashi 1 chome, Tsukuba-shi, Ibaraki-ken, 305, Japan] since May 28, 1996 (date of original deposition). The transformant stably retains the restriction endonuclease gene, despite 5 the absence of the AccIII modification enzyme gene therein. By the above-described method, a restriction endonuclease gene can be isolated without the co-presence of a modification enzyme gene that constitutes a restriction modification system gene.

10 The fourth aspect of the present invention provides a polypeptide possessing an activity of the AccIII restriction endonuclease.

In the present specification, the term "AccIII restriction endonuclease" as a general term of 15 polypeptides possessing an activity of the AccIII restriction endonuclease may be used in some cases. The AccIII restriction endonuclease of the present invention comprises the amino acid sequence described in SEQ ID NO:1 in the Sequence Listing, for example.

20 The AccIII restriction endonuclease of the present invention also includes, but not limited to the above, the polypeptide containing the entire or a portion of the amino acid sequence described in SEQ ID NO:1 in the Sequence Listing and possessing an activity of the AccIII 25 restriction endonuclease. Furthermore, the polypeptide

having an amino acid sequence resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:1 or a portion thereof in the Sequence Listing and possessing an activity of the AccIII restriction endonuclease is included in the scope of the present invention.

Generally, a naturally-occurring protein can undergo deletion, insertion, addition, substitution and other variations of amino acid residues in its amino acid sequence due to modifications, etc. of the protein in vivo or during purification, as well as those due to polymorphism and variation of the gene encoding it.

Nevertheless, it is known that there are some such polypeptides which are substantially equivalent to variation-free proteins in terms of physiological and biological activity. Thus, those structurally different from the corresponding protein, but having no significant difference of function or activity from the protein is within the scope of the present invention. It is also the same when artificially introducing the above variations to the amino acid sequence of a protein and in this case, it is possible to produce more diverse variants. For example, the methionine residue at the N-terminus of a

protein expressed in *Escherichia coli* is reportedly often removed by the action of methionine aminopeptidase, but the removal is not completely done depending on the kinds of proteins, and some such expressed proteins have the 5 methionine residue and others not. However, the presence or absence of the methionine residue does not affect protein activity in most cases. It is also known that a polypeptide resulting from replacement of a particular cysteine residue with serine in the amino acid sequence of 10 human interleukin 2 (IL-2) retains IL-2 activity [Science, 224, 1431 (1984)].

In addition, in producing a protein by gene engineering, the desired protein is often expressed as a fused protein. For example, the N-terminal peptide chain 15 derived from another protein is added to the N-terminus of the desired protein to enhance the expression of the desired protein, or purification of the desired protein is facilitated by adding an appropriate peptide chain to the N- or C-terminus of the desired protein, expressing the protein, and using a carrier showing affinity for the peptide chain added. Thus, even if the polypeptide has an amino acid sequence partially different from the AccIII restriction endonuclease of the present invention, it is within the scope of the present invention as long as it 20 possesses essentially equivalent activity to the AccIII 25

restriction endonuclease of the present invention.

The AccIII restriction endonuclease can be obtained by, for example, culturing the above-described transformant *Escherichia coli* JM109/pCRA19, which contains 5 the AccIII restriction endonuclease gene, adding the inducing agent IPTG at an appropriate time during its cultivation to increase the copy number of pCRA19, and subsequently infecting with the phage M13sp6.

The AccIII restriction endonuclease can be harvested 10 from the transformant culture by, for example, collecting cells from the culture, subsequently extracting the enzyme by ultrasonic disruption, ultracentrifugation, etc., and then purifying the enzyme by a combination of nucleic acid removal, salting-out, affinity chromatography, gel 15 filtration, ion exchange chromatography, etc. Because the above-described culture, which serves as the starting material for this purification, does not contain other restriction endonucleases, such as AccI and AccII, an enzyme preparation of desired purity can be obtained more 20 easily than by conventional purification methods.

The fifth aspect of the present invention provides a DNA encoding a polypeptide possessing an activity of the AccIII restriction endonuclease.

The DNA of the present invention encoding a 25 polypeptide possessing an activity of the AccIII

restriction endonuclease comprises a DNA encoding the amino acid sequence described in SEQ ID NO:1 in the Sequence Listing, and includes but not limited to, a DNA comprising the base sequence described in SEQ ID NO:2 in the Sequence Listing, for example. Specifically, the following DNAs are within the scope of the present invention.

- (1) a DNA encoding a polypeptide which contains the entire or a portion of the amino acid sequence described in SEQ ID NO:1 in the Sequence Listing and possesses an activity of the AccIII restriction endonuclease;
- (2) a DNA containing the entire or a portion of the DNA shown in SEQ ID NO:2 in the Sequence Listing, wherein the expression product of the DNA possesses an activity of the AccIII restriction endonuclease;
- (3) a DNA encoding a polypeptide resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:1 in the Sequence Listing or a portion thereof and possessing an activity of the AccIII restriction endonuclease; and
- (4) a DNA capable of hybridizing to the DNA described in above (1) to (3), and encoding a polypeptide possessing an activity of the AccIII restriction endonuclease, etc..

In addition, if the hybridization is carried out

under the stringent condition using the above obtained DNA as a probe, a similar DNA somewhat different from the obtained DNA (SEQ ID NO:2 in the Sequence Listing) but encoding a polypeptide possessing the same enzyme 5 activity, can be obtained. Such a DNA is also included in the scope of the present invention.

Such a stringent condition refers to that the membrane with DNA immobilized thereon is subjected to 10 hybridization with the probe in a solution containing 6 × SSC (1 × SSC is a solution of 8.76 g of NaCl and 4.41 g of sodium citrate in 1 liter of water), 1% SDS, 100 µg/ml salmon sperm DNA, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone and 0.1% Ficoll, incubating at 65°C 15 for 20 hours, for example.

Methods for obtaining similar DNA encoding the AccIII restriction endonuclease by hybridization include, for example, the following method.

First, DNA obtained from an appropriate gene source 20 is ligated to a plasmid or a phage vector by a conventional method to yield a DNA library. This library is introduced into an appropriate host; the resulting transformants are cultured on plates; colonies or plaques that have grown are transferred onto nitrocellulose or 25 nylon membranes and denatured, after which the DNA is

fixed onto the membrane. These membranes are incubated for hybridization in a solution of the above-described composition containing a probe previously labeled with  $^{32}\text{P}$  etc. (probe used may be any polynucleotide encoding the entire or a portion of the amino acid sequence shown by SEQ ID NO:1 in the Sequence Listing, exemplified by a polynucleotide consisting of, or containing, the entire or a portion of the base sequence shown by SEQ ID NO:2 in the Sequence Listing) under the conditions shown above. After completion of the hybridization, the non-specifically adsorbed probe is washed out, followed by autoradiography etc., to identify clones that have hybridized to the probe. This procedure is repeated until the desired hybridizing clone is isolated. The clone thus obtained retains DNA encoding a polypeptide having the desired enzyme activity.

The DNA obtained is determined for base sequence to confirm that it encodes the desired enzyme protein, by, for example, the method described below.

For base sequencing, the transformant is cultured in a test tube etc. when the host is *Escherichia coli*, and a plasmid is prepared by a conventional method, provided that the transformant has been prepared using a plasmid vector. Using the plasmid obtained as a template as is, or after the insert is taken out and subcloned into the

M13 phage vector etc., the base sequence is determined by the dideoxy method. In the case of a transformant prepared using a phage vector as well, the base sequence can be determined by basically the same procedures.

5 These basic experimental processes from cultivation to base sequencing are described in, for example, Molecular Cloning: A Laboratory Manual, 1982, T. Maniatis et al., published by Cold Spring Harbor Laboratory.

10 Whether or not the DNA obtained is similar DNA encoding the desired AccIII restriction endonuclease can be confirmed by comparing the determined base sequence with the base sequence shown by SEQ ID NO:2 in the Sequence Listing, or by comparing the amino acid sequence deduced from the determined base sequence with the amino 15 acid sequence shown by SEQ ID NO:1 in the Sequence Listing.

When the DNA obtained does not contain the entire portion of the region encoding the desired restriction endonuclease, the entire encoding region can be obtained 20 by synthesizing a primer on the basis of the base sequence of the DNA obtained, and amplifying the lacking region by PCR using the primer, or repeating screening the DNA library using the DNA fragment obtained as a probe.

It is possible to prepare a transformant containing 25 the thus-obtained similar DNA encoding the AccIII

restriction endonuclease, to allow the transformant to express the enzyme protein encoded by the DNA, and to purify the enzyme protein expressed. Preparation of the transformant and expression and purification of the enzyme 5 protein can be all achieved using the plasmid of the present invention. The enzyme protein thus obtained retains AccIII restriction endonuclease activity.

Additionally, the AccIII modification enzyme and DNA encoding the enzyme, both of which have not been obtained 10 so far, can also be obtained using the above-described DNA encoding the AccIII restriction endonuclease.

For example, on the basis of the mutually close location of a restriction endonuclease gene and a modification enzyme gene in many cases, this purpose can 15 be accomplished by obtaining a gene region encoding a protein near the restriction endonuclease gene by a DNA amplification reaction with a cassette library as a template, inserting it into an appropriate expression vector to allow the gene to be expressed, and confirming 20 AccIII modification enzyme activity by an appropriate method. AccIII modification enzyme activity can also be confirmed, for example, on the basis of the resistance of the DNA prepared from the transformant to the cleavage activity of the AccIII restriction endonuclease. Provided 25 that the base sequence of the above-described gene region

is determined to confirm homology to a conserved region between modification enzyme genes of a known restriction modification system [Journal of Molecular Biology, Vol. 206, pp. 305-321 (1989)], it can be anticipated to some 5 extent before gene expression that the gene is a modification enzyme gene.

By using a cassette library of the genomic DNA of the Acc bacterium as described above, the AccIII modification enzyme and DNA encoding it can be obtained. Its amino 10 acid sequence and base sequence are shown by SEQ ID NO:13 and SEQ ID NO:14 in the Sequence Listing, respectively.

AccIII modification enzyme herein is not limited to the above described. As stated in the description of AccIII restriction endonuclease, it is a polypeptide 15 containing the entire or a portion of the amino acid sequence described in SEQ ID No:13 in the Sequence Listing and possessing the AccIII modification enzyme activity. Furthermore, the polypeptide resulting from at least one of deletion, addition, insertion or substitution of one or 20 more amino acid residues in the amino acid sequence of SEQ ID NO:13 or a portion thereof in the Sequence Listing and possessing the AccIII modification enzyme activity is also included in the scope of the present invention.

A DNA encoding AccIII modification enzyme in the 25 present invention herein comprises a DNA encoding the

amino acid sequence described in the SEQ ID NO:13 in the Sequence Listing, and includes, but not limited to, a DNA comprising the base sequence described in the SEQ ID NO:14 in the Sequence Listing, for example. Specifically, the  
5 following DNAs are within the scope of the present invention.

- (1) a DNA encoding a polypeptide containing the entire or a portion of the amino acid sequence described in SEQ ID NO:13 in the Sequence Listing and possessing the AccIII modification enzyme activity;  
10 (2) a DNA containing the entire or a portion of the DNA shown in SEQ ID NO:14 in the Sequence Listing, wherein the expression product of the DNA possesses the AccIII modification enzyme activity;  
15 (3) a DNA encoding a polypeptide resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:13 or a portion thereof in the Sequence Listing and possessing the AccIII modification enzyme activity; and  
20 (4) a DNA capable of hybridizing to the DNA described in above (1) to (3), and encoding a polypeptide possessing the AccIII modification enzyme activity, etc..

25 The present invention is hereinafter described in

more detail by means of the following reference example  
and working examples, which examples are not to be  
construed as limitative. Of the procedures described  
herein, basic ones regarding plasmid preparation,  
5 restriction endonuclease digestion, etc. were achieved in  
accordance with the methods described in Molecular  
Cloning: A Laboratory Manual, 2nd edition, edited by T.  
Maniatis et al., published by Cold Spring Harbor  
Laboratory, 1989.

10

Reference Example

(1) Culture medium and conditions

*Escherichia coli* was aerobically cultured at 37°C  
using LB medium (1% tryptone, 0.5% yeast extract, 0.5%

15 NaCl, pH 7.0). Antibiotics were each added to the medium  
at various concentrations depending on the plasmid  
retained by the *Escherichia coli* as follows: 25 µg/ml  
kanamycin for pFSP6, 20 µg/ml ampicillin for pXX325, 50  
µg/ml ampicillin for ampicillin-resistant ColE1 type  
20 plasmid, and 100 µg/ml ampicillin for ampicillin-resistant  
pUC type plasmid. In the expression induction experiment,  
the culture broth obtained after cultivation until the  
stationary phase was inoculated to a fresh medium at 1%,  
then aerobically cultured at 37°C, after which  
25 isopropyl-β-D-thiogalactoside (IPTG) was added to a final

concentration of 0.2 mM upon reach of an OD<sub>600</sub> value of 0.6 (6 x 10<sup>8</sup> cells/ml), followed by further cultivation.

(2) Construction of the system plasmid pFSP6

5       The system plasmid pFSP6 was constructed with *Escherichia coli* JM109 as a host, according to the procedure directed in Figure 8. The plasmid pSP6-2 [Nucleic Acids Research, Vol. 15, pp. 2653-2664 (1987)] was digested with HindIII (produced by Takara Shuzo) and  
10      blunted at both ends, after which it was further digested with BamHI (produced by Takara Shuzo) to yield an about 2.8 kb DNA fragment containing the SP6 RNA polymerase gene, which fragment was mixed with the plasmid vector pUC18 (produced by Takara Shuzo), previously digested with  
15      BamHI and HincII (produced by Takara Shuzo), for ligation to construct the plasmid pUCSP. Next, the plasmid pMJR1560 [Gene, Vol. 51, pp. 225-267 (1987)] was digested with KpnI and blunted at both ends, after which it was further digested with PstI (produced by Takara Shuzo) to  
20      yield an about 1.3 kb DNA fragment containing the lacIq gene, which was then isolated. The above plasmid pUCSP was digested with HindIII and blunted at both ends, after which it was further digested with PstI to yield a DNA fragment, which was mixed with the above-described about  
25      1.3 kb DNA fragment for ligation to construct the plasmid

pUCSPlac.

Furthermore, an about 1.5 kb DNA fragment obtained by PstI digestion of pUCKm [Journal of Molecular Biology, Vol. 147, pp. 217-226 (1981)], which contains the 5 kanamycin resistance gene, was introduced into the PstI site of the above-described plasmid pUCSPlac to construct the plasmid pUCSPlacKm. The plasmid was digested with AatII (produced by Toyobo), after which it was partially digested with NspI (produced by Takara Shuzo) to isolate 10 an about 6.5 kb DNA fragment, which was then blunted at both ends.

On the other hand, the plasmid pXX325 [Proceedings of the National Academy of Sciences of the USA, Vol. 80, pp. 4784-4788 (1983)] was digested with HindIII and blunted at 15 both ends, after which it was further digested with SalI to isolate an about 6.8 kb DNA fragment containing the replication origin of the miniF plasmid, which fragment was then mixed with the above-described about 6.5 kb DNA fragment for ligation to construct the plasmid pFSP6. 20 *Escherichia coli* HB101 was transformed with the plasmid to form *Escherichia coli* HB101/pFSP6.

(3) Construction of multicopy model expression plasmids

The  $P_{SP6}$  linker, a double-stranded oligonucleotide 25 containing the minimum region of the SP6 promoter, was

prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:15 and SEQ ID NO:16 in the Sequence Listing, respectively, and was mixed with the plasmid pMS434 [Gene, Vol. 57, pp. 89-99 (1987)], previously digested with XhoI (produced by Takara Shuzo) and HindIII, for ligation to construct the model expression plasmid pMSP6L inserted the above-described promoter sequence upstream of the  $\beta$ -galactosidase gene on the plasmid (Figure 1).

On the other hand, by inserting a DNA fragment containing the inherent SP6 promoter sequence, obtained by digesting pSP64 [Nucleic Acids Research, Vol. 12, pp. 7035-7056 (1984)] with AccII and HindIII, into the XhoI-HindIII site of the above-described plasmid pMS434, the model expression plasmid pMSP6F was constructed (Figure 1). Furthermore, the  $P_{SP6}$ - $O_{lac}$  linker, which contains the minimum region of the SP6 promoter and the lac operator region, was prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:17 and SEQ ID NO:18 in the Sequence Listing, respectively, and was inserted between the XhoI-HindIII site of the above-described plasmid pMS434 to construct the model expression plasmid pMSP60 (Figure 2).

(4) Promoter activity of the SP6 promoter for *Escherichia*

*coli* RNA polymerase

The plasmids pMSP6L and pMSP6F constructed in Reference Example (3) and the plasmid pMS434 as a control, which does not contain the SP6 promoter sequence, were 5 each introduced into the *Escherichia coli* shown in Table 1. The resulting transformants were each cultured by the method described in Reference Example (1), after which each cell culture broth was harvested during the logarithmic growth phase. The OD<sub>600</sub> value of each culture 10 broth collected was determined with a portion thereof, while the remaining portion was transferred to a pre-cooled test tube and supplemented with chloramphenicol to a final concentration of 100 mg/ml. With this culture broth as a sample,  $\beta$ -galactosidase activity was determined 15 by the method described in Experiments in Molecular Genetics, edited by J.H. Miller, published by Cold Spring Harbor Laboratory, 1972.

As shown in Table 2, *Escherichia coli* MC4100 incorporating the model expression plasmid pMSP6L or 20 pMSP6F, both harboring a sequence containing the SP6 promoter, exhibited similar level of low  $\beta$ -galactosidase activity, irrespective of the promoter sequence inserted. However, that incorporating the plasmid pMS434, which does 25 not contain the SP6 promoter, exhibited only lower enzyme activity. In addition, comparing *Escherichia coli* MRi7

and MRi80 as the hosts revealed decreased  $\beta$ -galactosidase activity in MRi80, wherein the copy number of the plasmid was also decreased.

5       (5) Evaluation of the system plasmid pFSP6

Each transformant prepared in Reference Example (4) into which the system plasmid pFSP6 was further introduced, was cultured by the method described in Reference Example (1);  $\beta$ -galactosidase activity was determined both in a non-inductive condition and in an inductive condition before and after IPTG addition. The results were shown in Table 3. All transformants exhibited  $\beta$ -galactosidase activity in a non-inductive condition at a level similar to that obtained in the absence of the plasmid pFSP6 shown in Table 2.

On the other hand, with induction by IPTG addition, the  $\beta$ -galactosidase activity in the transformants incorporating the plasmids pMSP6L and pMSP6F was 18 to 32 times compared with that obtained in a non-inductive condition, while there was no increase in the activity in the transformants incorporating the plasmid pMS434.

(6) Effect of the lac operator sequence on expression control

25       Transformants obtained by introducing the plasmids

pMSP6L, pMSP6F, pMSP6O and pMS434, respectively, into *Escherichia coli* MC4100 incorporating the plasmid pFSP6 were each cultured by the method described in Reference Example (1), and  $\beta$ -galactosidase activity in a non-inductive condition was determined. The results are shown in Table 4. The transformant incorporating the plasmid pMSP6O, which contains the lac operator sequence, still showed some  $\beta$ -galactosidase activity, although the activity level was lower than those obtained with the plasmids pMSP6L and pMSP6F. In short, expression in a non-inductive condition could not be completely suppressed simply by introduction of the lac operator sequence.

Table 4

Strain	$\beta$ - Galactosidase Activity (Non-Inductive Condition)			
	pMSP6F	pMSP6L	pMSP6O	pMS434
MC4100(pFSP6)	221	234	30	25

20

Example 1

(1) Culture medium and conditions

*Escherichia coli* was aerobically cultured at 37°C using LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0). Antibiotics were each added to the medium

at various concentrations depending on the plasmid retained by the *Escherichia coli* as follows: 25 µg/ml kanamycin for pFSP6, 50 µg/ml ampicillin for ampicillin-resistant ColE1 type plasmid, 100 µg/ml ampicillin for ampicillin-resistant pUC type plasmid, 5 30 µg/ml chloramphenicol for chloramphenicol-resistant runaway plasmid and 30 µg/ml ampicillin for ampicillin-resistant runaway plasmid. In the expression induction experiment, the culture broth obtained after cultivation until the stationary phase was inoculated to a fresh medium at 1%, then aerobically cultured at 37°C, after which isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.2 mM upon reach of an OD<sub>600</sub> value of 0.6 ( $6 \times 10^8$  cells/ml), followed by further cultivation.

15

(2) Construction of the runaway plasmid pHs2870

The runaway plasmid pHs2870, which provided a basis for construction of the expression plasmid, was constructed by the procedures shown in Figure 3. The 106 NdeI DNA fragment prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:3 and SEQ ID NO:4 in the Sequence Listing, respectively, was mixed with the plasmid vector pUC19, previously digested with NdeI, for ligation. The plasmid pUC106A thus obtained was digested 20 with PvuII and subjected to self-ligation to yield the 25

yield the plasmid pUC106AdPO. With this plasmid  
pUC106AdPO as a template, PCR was then conducted using the  
RNAlIA primer (whose base sequence shown by SEQ ID NO:5 in  
the Sequence Listing) and the 1870 primer (whose base  
5 sequence shown by SEQ ID NO:6 in the Sequence Listing), to  
yield an amplified DNA fragment, which was then digested  
with XbaI and mixed with the plasmid pSTV28, previously  
digested with XbaI, for ligation to yield the plasmid  
pHS2870. The construct of the plasmid pHS2870 is shown in  
10 Figure 4.

(3) Construction of the runaway plasmid pCRS04

After digestion with AccI (produced by Takara Shuzo)  
and NspI, the above-described plasmid pHS2870 was blunted  
15 at both ends and subjected to self-ligation to yield the  
plasmid pCRS01, which lacks the P15A replication origin.  
The plasmid was then digested with EcoRI (produced by  
Takara Shuzo) and XbaI, after which it was blunted at both  
ends and subjected to self-ligation in the same manner as  
20 above to construct the plasmid pCRS02. After an about 1.2  
kb DNA fragment obtained by digesting the plasmid pMJR1560  
with KpnI (produced by Takara Shuzo) and PstI was blunted  
at both ends, it was mixed with the above-described  
plasmid pCRS02, previously digested with NspV and VspI  
25 (both produced by Takara Shuzo) and then blunted at both

ends, for ligation to yield the plasmid pCRS04. The flow diagram of the construction of the plasmid pCRS04 is shown in Figure 5, and the construct of the plasmid pCRS04 shown in Figure 6.

5

(4) Construction of the runaway expression vector pACE601

The above-described plasmid pCRS04, previously digested with NheI (produced by Takara Shuzo) and subsequently blunted at both ends, was mixed with the 10  $P_{SP6}$ -O<sub>lac</sub> EX linker, prepared from the two DNA strands whose base sequences are shown by SEQ ID NO:7 and SEQ ID NO:8 in the Sequence Listing, for ligation to construct the plasmid pACE601. The flow diagram of the construction of the plasmid pACE601 is shown in Figure 9. The 15 construction of these plasmids were conducted with *Escherichia coli* JM109 as a host.

(5) Construction of the runaway expression vector pACE611

The runaway expression vector pACE601 was digested 20 with XhoI-HindIII, and the  $P_{SP6}$  linker, consisting of the synthetic oligo-DNAs shown by SEQ ID NO:15 and SEQ ID NO:16 in the Sequence Listing, was inserted into that site to construct the runaway expression vector pACE611. The construct of pACE611 is shown in Figure 10. The flow 25 diagram of the construction of the plasmid pACE611 is

shown in Figure 11.

(6) Construction of the runaway expression vectors

pACE701 and pACE702

An about 1 kb DNA fragment obtained by digesting the plasmid vector pUC118 (produced by Takara Shuzo) with BspHI (produced by NEB) was blunted at both ends, after which it was mixed with the above-described plasmid pCRS04, previously digested with NheI, and blunted at both ends, for ligation to construct the plasmid pCRS70. The flow diagram of the construction of the plasmid pCRS70 is shown in Figure 12. Next, the plasmid was digested with NcoI (produced by Takara Shuzo) and BsaAI (produced by NEB), after which it was blunted at both ends and mixed with the above-described  $P_{SP6}$ -O<sub>lac</sub> EX linker for ligation to construct two plasmids pACE701 and pACE702, which incorporate the linker inserted in mutually opposite directions. The flow diagrams of the construction of the plasmids pACE701 and pACE702 are shown in Figure 13. The construction of these plasmids were conducted with *Escherichia coli* JM109 as a host.

(7) Construction of model runaway expression plasmids

PCR was conducted using the primers trpA-N-NcoI and lacZ-C-NcoI with the above-described plasmid pMS434 as a

template, to yield a DNA fragment containing the β-galactosidase gene. The base sequences of the primers trpA-N-NcoI and lacZ-C-NcoI are shown by SEQ ID NO:9 and SEQ ID NO:10 in the Sequence Listing, respectively. After 5 digestion with NcoI, the fragment was mixed with each of the above-described plasmids pACE601, pACE701 and pACE702, all previously digested with NcoI, for ligation to yield the model runaway expression plasmids pACE601Z, pACE701Z and pACE702Z, all incorporating the β-galactosidase gene 10 introduced downstream of the  $P_{SP6}-O_{lac}$  sequence. The construction of these plasmids were conducted with *Escherichia coli* JM109 as a host.

(8) Evaluation of the expression levels of runaway 15 expression plasmid in a non-inductive condition  
The transformants MC4100/pFSP6/pACE601Z, MC4100/pFSP6/pACE701Z and MC4100/pFSP6/pACE702Z, which were obtained after introducing the above-described model runaway expression plasmids pACE601Z, pACE701Z and 20 pACE702Z, respectively into *Escherichia coli* MC4100 incorporating the above-described system plasmid pFSP6 (hereinafter referred to as MC4100/PFSP6), were each cultured under the conditions described in Example (1); each culture broth collected during the logarithmic growth 25 phase was assayed for β-galactosidase activity by the

method described in Reference Example (4). In all transformants examined,  $\beta$ -galactosidase activity was below the detection limit, demonstrating a greater expression-suppressing effect than that obtained with the 5 multicopy plasmids shown in Reference Example (4).

(9) Construction of the Nsp7524 III restriction endonuclease gene expression plasmid

The plasmid pBRN3, which contains the Nsp7524 III restriction modification system gene, was prepared from 10 *Escherichia coli* MC1061/pBRN3 (FERM BP-5741). PCR was conducted using the primers L-ORF and NspR-ORF3 with this plasmid as a template to yield an about 1 kb DNA fragment containing the Nsp7524 III restriction endonuclease gene 15 alone. The base sequences of the primers L-ORF and NspR-ORF3 are shown by SEQ ID NO:11 and SEQ ID NO:12 in the Sequence Listing, respectively. After digestion with NcoI, the fragment was mixed with the above-described plasmid pACE601, previously digested with NcoI, for 20 ligation to construct the plasmid pACE601-NspIII, which incorporates the Nsp7524 III restriction enzyme gene alone introduced downstream of the  $P_{SP6}$ - $O_{lac}$  sequence. The plasmid was stably retained in *Escherichia coli* JM109 not containing the Nsp7524 III modification enzyme gene.

(10) Construction of Nsp7524 III restriction endonuclease gene expression system

The transformants HB101/pFSP6/pACE601-NspIII and HB101/pFSP6/pACE601, which were obtained after introducing 5 the above-described plasmid pACE601-NspIII, which contains the Nsp7524 III restriction endonuclease gene, and the control plasmid pACE601 into *Escherichia coli* HB101 incorporating the above-described system plasmid pFSP6 (hereinafter referred to as HB101/PFSP6), were each 10 cultured in LB medium until the stationary phase; each culture broth collected was inoculated to two tubes of fresh medium at 1% and aerobically cultured at 37°C. Upon reach of an OD<sub>600</sub> value of 0.6, IPTG was added to one of the tubes to a final concentration of 0.2 mM, followed by 15 further cultivation. After completion of the cultivation, cells were harvested, suspended in cell disruption buffer A (20 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol), and disrupted by ultrasonication, followed by centrifugation to yield a crude extract.

20 A 1 µl portion of this crude extract was added to 30 µl of a reaction mixture (10 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 50 mM NaCl, 1 µg λ-DNA) and reacted at 37°C for 1 hour, after which the reaction mixture was subjected to agarose gel electrophoresis to examine for 25 the digestion of λ-DNA and confirm restriction

endonuclease activity. As shown in Figure 14, restriction endonuclease activity was observed only in HB101/pFSP6/pACE601-NspIII with expression induced by the addition of IPTG, and its  $\lambda$ -DNA cleavage pattern agreed 5 with that of AvaI, an isoschizomer of Nsp7524 III.

The results of the above-described  $\lambda$ -DNA digestion reaction as conducted for extended periods of 2 and 3 hours are shown in Figure 15. In this case, because the crude extract used in the present experiment contained 10 nuclease derived from the host *Escherichia coli*, the DNA fragment resulting from Nsp7524 III activity underwent further degradation by *Escherichia coli* nuclease, resulting in bands whose density decreased as the increase in reaction time, on lanes 8 through 10, with no bands 15 detected on lane 10. On lanes 11 through 13, the Nsp7524 III digestion fragment was not produced because of the absence of Nsp7524 III induction even when reaction time was extended, demonstrating that the  $\lambda$ -DNA was made into the lower molecular weight fragments by the direct action 20 of *Escherichia coli* nuclease. No restriction endonuclease activity was detected in the crude extract from HB101/pFSP6/pACEA601.

Example 2

25 Isolation and expression of the AccIII restriction

endonuclease gene

(1) Determination of N-terminal amino acid sequence of AccIII restriction endonuclease protein and synthesis of primer DNA corresponding to the amino acid  
5 sequence

About 2 ku of a commercial product of the AccIII restriction endonuclease (produced by Takara Shuzo) was subjected to gel filtration using a column of Sephadryl S300 (produced by Pharmacia) to determine the molecular weight of the AccIII restriction endonuclease. Judging from the elution position where activity was detected, the molecular weight of the AccIII restriction endonuclease was proven to be about 70,000. Because most restriction endonuclease proteins are dimers, the AccIII restriction endonuclease protein was expected to be mobilized to a position for a molecular weight of about 35,000 in SDS polyacrylamide gel electrophoresis.  
10  
15

Next, to obtain a sample for N-terminal amino acid sequencing of the AccIII restriction endonuclease protein, about 2 ku of a commercial product of the AccIII restriction endonuclease, together with a protein molecular weight marker, was subjected to SDS polyacrylamide gel electrophoresis, after which the protein was transferred from the gel to a PVDF membrane  
20  
25 and stained with bromophenol blue to confirm the protein

position. After destaining with 10% acetic acid-50% methanol, the portion of the PVDF membrane where a protein of about 35,000 molecular weight was blotted was cut out and subjected to automatic Edman degradation using the 5 protein sequencer G1000A (produced by Hewlett-Packard) to determine the N-terminal amino acid sequence shown by SEQ ID NO:19 in the Sequence Listing. On the basis of this sequence, AccIII primers 1 and 2, shown by SEQ ID NO:20 and SEQ ID NO:21 in the Sequence Listing, respectively, 10 were then synthesized for use as a pair of cassette primers.

(2) Preparation of genomic DNA of Acc bacterium

In accordance with the method of Kita et al., 15 described in Nucleic Acids Research, Vol. 13, pp. 8685-8694 (1985), the Acc bacterium was cultured to obtain wet cells. Two grams of the wet cells obtained was suspended in 10 ml of buffer B [25 mM Tris-HCl (pH 8.0), 50 mM glucose, 10 mM EDTA], stirred in the presence of 1 20 ml of a lysozyme solution prepared to 2 mg/ml in buffer B, and kept standing at 37°C for 20 minutes. Next, 28 ml of buffer C [100 mM NaCl, 100 mM Tris-HCl (pH 8.0)] was added to this solution, followed by stirring. One milliliter of a proteinase K solution prepared to 20 mg/ml in TE [10 mM 25 Tris-HCl, 1 mM EDTA (pH 8.0)] and 4 ml of a 10% SDS

solution were further added, followed by stirring, after which the mixed solution was kept standing at 37°C for 1 hour.

To this solution, 6 ml of a 5 M aqueous solution of  
5 NaCl and 6 ml of buffer D [10% CTAB (cetyl trimethyl ammonium bromide), 0.7 M NaCl] was added, followed by stirring, after which the mixed solution was kept standing at 60°C for 20 minutes. This solution was treated with phenol and subsequently with chloroform, after which the  
10 water layer was separated. To the water layer, 50 µl of an RNaseA (produced by Sigma) solution prepared to 10 mg/ml in TE was added, followed by stirring, after which the mixed solution was kept standing at 37°C for 40 minutes. After being kept standing, this solution was  
15 treated with phenol and subsequently with chloroform, after which the water layer was separated. To the water layer, an equal volume of cold ethanol was added; the DNA precipitated was recovered by winding around a glass capillary. The DNA was washed with 70% ethanol and  
20 dissolved in 3 ml of TE to yield about 200 µg of genomic DNA.

(3) Preparation of Acc genomic cassette library

The following procedures were conducted basically in  
25 accordance with the method described on pages F16-F17 in

"Gene Engineering Guide" (1995-1996 edition), Takara Shuzo.

The genomic DNA obtained in (2) was completely digested with EcoRI (produced by Takara Shuzo); the DNA fragment obtained was ligated with an EcoRI cassette (produced by Takara Shuzo), having a cohesive end complementary thereto, to yield an EcoRI cassette library. Similarly, the genomic DNA was completely digested separately with the restriction endonucleases BglIII, 10 EcoT14I, EcoT22I, HindIII, PstI, SalI and XbaI (all produced by Takara Shuzo). The genomic DNA fragments obtained were each bound to each of several cassettes (produced by Takara Shuzo), having a complementary protruding end, to yield the BglIII, EcoT14I, EcoT22I, 15 HindIII, PstI, SalI and XbaI cassette libraries, respectively. These cassette libraries are generically referred to as the Acc genomic cassette library.

(4) Analysis of the AccIII restriction endonuclease gene  
20 A PCR-based DNA amplification reaction was carried out using a primer pair of AccIII primer 1 and cassette primer C1 (produced by Takara Shuzo) with the EcoRI cassette library obtained in (3) as a template. To efficiently and specifically amplify the desired region, a 25 second PCR-based DNA amplification reaction was carried

out using a primer pair of AccIII primer 2 and cassette primer C2 (produced by Takara Shuzo) with a portion of the reaction liquid obtained, to yield an amplified DNA fragment. Similarly, with the BglIII, EcoT14I, EcoT22I, HindIII, PstI, SalI and XbaI cassette libraries as a template respectively, the above two-step PCR-based DNA amplification reaction was carried out separately to yield amplified DNA fragments. Each amplified DNA was analyzed by agarose gel electrophoresis; the amplified DNA fragment derived from the XbaI cassette library showed a particularly high amplification efficiency.

With this in mind, a PCR-based DNA amplification reaction was again carried out using a primer pair of AccIII primer 1 and cassette primer C1 with the XbaI cassette library as a template. This reaction mixture was subjected to agarose gel electrophoresis; an about 0.5 kb amplified DNA fragment was recovered from the gel. A second PCR-based DNA amplification reaction was carried out using a primer pair of AccIII primer 2 and cassette primer C2 with the DNA fragment obtained as a template. Base sequencing of the about 0.5 kb amplified DNA fragment obtained demonstrated that the fragment encodes a portion of the protein whose N-terminal amino acid sequence was determined in (1). On the other hand, the putative molecular weight of the AccIII restriction endonuclease

protein is about 35,000, and the gene encoding the protein is assumed to be about 1 kb in length. It was therefore expected that the base sequence of the AccIII restriction endonuclease gene could be determined, provided that  
5 information on the accurate base sequence of the 5'-terminal region to which AccIII primers 1 and 2 annealed, and information on the remaining about 0.5 kb base sequence in the 3' region, were available, in addition to the above-described information on the about  
10 0.5 kb base sequence.

With this in mind, to determine the accurate base sequence of the 5'-terminal region of the AccIII restriction endonuclease gene region, AccIII primer 3, shown by SEQ ID NO:22 in the Sequence Listing, and AccIII primer 4, shown by SEQ ID NO:23 in the Sequence Listing,  
15 were synthesized. Next, a PCR-based DNA amplification reaction was carried out using a primer pair of AccIII primer 4 and cassette primer C1 with the EcoRI cassette library obtained in (3) as a template. A PCR-based DNA amplification reaction was carried out using a primer pair  
20 of AccIII primer 3 and cassette primer C2 with a portion of this reaction mixture. Similarly, with the BglIII, EcoT14I, EcoT22I, HindIII, PstI, SalI and XbaI cassette libraries as a template respectively, the above two-step  
25 PCR-based DNA amplification reaction was separately

carried out. Of the DNA fragments amplified, the shortest, i.e., the about 1 kb DNA fragment was obtained with the EcoT14I cassette library as a template, the base sequence thereof was determined.

5 Furthermore, to determine the about 0.5 kb sequence on the 3' side of the gene region assumed to encode the AccIII restriction endonuclease protein, AccIII primer 5, shown by SEQ ID NO:24 in the Sequence Listing, and AccIII primer 6, shown by SEQ ID NO:25 in the Sequence Listing,  
10 were synthesized. A PCR-based DNA amplification reaction was carried out using a primer pair of AccIII primer 5 and cassette primer C1 with the EcoRI cassette library obtained in (3) as a template. A PCR-based DNA amplification reaction was carried out using a primer pair  
15 of AccIII primer 6 and cassette primer C2 with a portion of this reaction mixture. Similarly, with the BglII, EcoT14I, EcoT22I, HindIII, PstI, SalI and XbaI cassette libraries as a template respectively, the above two-step PCR-based DNA amplification reaction was carried out  
20 separately.

25 Of the DNA fragments amplified, the about 0.5 kb and about 0.8 kb DNA fragments each obtained with the EcoT22I and HindIII cassette libraries as a template respectively were subjected to a base sequencing. Combining the results for the three base sequences determined, the base

sequence information on an about 1.6 kb DNA fragment was obtained. Its base sequence is shown by SEQ ID NO:26 in the Sequence Listing. Furthermore, searching for an open reading frame (ORF) capable of encoding the protein  
5 demonstrated the presence of ORF1 at base numbers 558 through 1442, a portion thought to be ORF2 at base numbers 1588 through 1434, and a portion thought to be ORF3 at base numbers 1 through 535. Encoding the protein whose N-terminal amino acid sequence was determined in (1), ORF1  
10 was deemed to be the AccIII restriction endonuclease gene. The base sequence of the AccIII restriction endonuclease gene is shown by SEQ ID NO:2 in the Sequence Listing, wherein the fourth base, as counted from the 5' terminus, is C. The amino acid sequence deduced from the base  
15 sequence is shown by SEQ ID NO:1 in the Sequence Listing, wherein the second amino acid, as counted from the N terminus, is Leu. The direction of translation of ORF2 is opposite that of ORF1 and ORF3.

20 (5) Construction of the plasmid pCRA19

Next, a plasmid for expression of the AccIII restriction endonuclease gene was constructed. First, to obtain the gene, the primer Acc-RL, shown by SEQ ID NO:27 in the Sequence Listing, and the primer Acc-RR, shown by  
25 SEQ ID NO:28 in the Sequence Listing, were synthesized. A

restriction endonuclease NcoI recognition sequence site was introduced into both primers. Using the above described primer pair, it is possible to cut out the AccIII restriction endonuclease gene from an amplified DNA 5 fragment obtained by PCR with the genomic DNA of the Acc bacterium as a template, using the restriction endonuclease NcoI, and the translation codon frames coincide with each other under control of the SP6 promoter when the gene is inserted into the NcoI site of the 10 pACE611 vector. On the other hand, a use of this primer pair results in replacement of the fourth base, as counted from the 5' terminus of the AccIII restriction endonuclease gene, from C to G, and of the second amino acid, as counted from the N terminus of the protein 15 encoded by the gene, from Leu to Val. Using the above primer pair with the genomic DNA of the Acc bacterium obtained in (2) as a template, a PCR-based DNA amplification reaction was carried out. This DNA fragment was completely digested with the restriction endonuclease 20 NcoI (produced by Takara Shuzo), after which it was subjected to agarose gel electrophoresis; a DNA fragment of about 900 bp size was recovered. Next, this DNA fragment was inserted into the NcoI site of the pACE611 vector so that it was located downstream of the SP6 25 promoter.

This recombinant DNA was introduced into *Escherichia coli* JM109 to yield transformants. Thirty transformants were randomly selected, and each inoculated to 5 ml of an LB medium containing 30 µg/ml chloramphenicol and cultured at 37°C. When the OD<sub>600</sub> value of the cell culture broth reached 0.6, IPTG was added to a final concentration of 2 mM to increase the plasmid copy number, followed by further cultivation at 37°C for 2 hours, after which plasmid DNA was prepared from each culture. Each plasmid DNA obtained was simultaneously cleaved with the restriction endonucleases HindIII and XbaI, followed by confirmation of the length of the resulting DNA fragments by agarose gel electrophoresis; a plasmid thought to contain the gene region inserted in the right direction was detected. This plasmid, designated pCRA19, was introduced into *Escherichia coli* JM109 to yield a transformant, which was designated *Escherichia coli* JM109/pCRA19.

(6) Construction of expression system for the AccIII restriction endonuclease gene

First, the SP6 RNA polymerase gene was introduced into *Escherichia coli* JM109 to construct a phage vector allowing further expression of the gene. Specifically, by inserting an SP6 RNA polymerase gene fragment obtained by

BamHI-HindIII digestion of pSP6-2 into the BamHI-HindIII site within the multicloning site of a commercial product of the phage vector M13mp18 (produced by Takara Shuzo), the SP6 RNA polymerase expression phage M13sp6 was  
5 constructed.

Next, the transformant *Escherichia coli* JM109/pCRA19 was inoculated to 5 ml of an LB medium containing 30 µg/ml chloramphenicol and cultured at 37°C. When the OD<sub>600</sub> value of the cell culture broth reached 0.6, IPTG was added to a  
10 final concentration of 2 mM to increase the plasmid copy number, followed by further cultivation at 37°C for 2 hours. These cells were then infected with the phage M13sp6 to express the protein encoded by the AccIII restriction endonuclease gene, followed by further  
15 cultivation at 37°C for 16 hours. A 11 mg portion of the wet cells obtained was suspended in 180 µl of cell disruption buffer E [20 mM Tris-HCl (pH 7.5), 10 mM 2-mercaptoethanol], after which the cells were disrupted by ultrasonication, followed by centrifugation (18000 g,  
20 10 minutes) to separate the solid and liquid.

Determining the activity of the supernatant under the activity determination conditions shown on the data sheet attached to the AccIII restriction endonuclease produced by Takara Shuzo demonstrated the production of the AccIII restriction endonuclease in an amount of about 8000 units  
25

per gram of wet cells, a level about 16 times that obtained with the Acc bacterium, per unit weight of wet cells. Neither activity of restriction endonucleases other than AccIII nor AccIII modification enzyme activity was noted in the supernatant obtained. Regarding the 5 AccIII restriction endonuclease gene inserted into the plasmid pCRA19, it was demonstrated that the fourth base, as counted from the translation initiation base, was replaced from C to G, upon DNA amplification by PCR, resulting in the replacement of the second amino acid, as 10 counted from the N-terminus of the translated protein, from Leu to Val, and that the protein possesses AccIII restriction endonuclease activity.

An isolation/mass production system for the AccIII 15 restriction endonuclease gene in the absence of AccIII modification enzyme was thus developed.

(7) Isolation of the AccIII modification enzyme gene

Modification enzyme genes and restriction 20 endonuclease genes are often closely located. With this in mind, to determine the ORF2 region deduced in (4) above, the base sequence of an about 1.4 kb DNA fragment obtained using the EcoRI cassette library as a template, out of the amplified DNA fragments prepared to determine 25 the 3' region of the AccIII restriction endonuclease gene

in (4) above, was determined. Next, to determine the ORF3 region deduced in (4) above, the base sequence of an about 2.2 kb DNA fragment obtained using the BglII cassette library as a template, out of the amplified DNA fragments prepared to determine the 5'-terminal region of the AccIII restriction endonuclease gene in (4) above, was determined. Combining these base sequences and the base sequence of the about 1.6 kb DNA fragment containing the AccIII restriction endonuclease gene region determined in (4) resulted in the information on the about 4.2 kb base sequence shown by SEQ ID NO:29 in the Sequence Listing. The AccIII restriction endonuclease gene was located at base numbers 1913 through 2797, ORF2 at base numbers 3712 through 2789, and ORF3 at base numbers 691 through 1890. Of these ORFs, ORF2 proved to contain a portion highly homologous to the conserved region among modification enzymes in a known restriction modification system.

Next, to obtain the ORF2 region, an about 1.1 kb ORF2-containing portion was cut out using EcoT22I and HpaI (produced by Takara Shuzo) from an amplified DNA fragment obtained by two-step PCR using a primer pair of Acc primer 6 and cassette primer C1 and another primer pair of Acc primer 5 and cassette primer C2 in the respective steps, with the EcoRI cassette library as a template, and was inserted into the SmaI site downstream of the lac promoter

in the pUC118 vector. If this recombinant plasmid contains the AccIII modification enzyme gene, and if the AccIII modification enzyme can be expressed in *Escherichia coli*, the DNA in the culture of the transformant obtained by introducing this recombinant plasmid into *Escherichia coli* JM109 would undergo methylation by the AccIII modification enzyme and acquire resistance to cleavage by the AccIII restriction endonuclease.

Because the pUC118 vector used to construct this recombinant plasmid has no AccIII restriction endonuclease recognition sequence, however, it is inappropriate to use this recombinant plasmid by itself to confirm AccIII modification enzyme activity. On the other hand, there is an AccIII restriction endonuclease recognition sequence in the plasmid pSTV29, which can be co-present with this recombinant plasmid in *Escherichia coli* JM109. With this in mind, to utilize pSTV29 as an index of expression of the AccIII modification enzyme, the above recombinant plasmid and pSTV29 were both introduced into *Escherichia coli* JM109 to yield transformants. Three transformants were each cultured at 37°C for 16 hours in 2 ml of an LB medium containing 100 µg/ml ampicillin, 30 µg/ml chloramphenicol and 2 mM IPTG, after which plasmid DNA was prepared from each culture.

The DNA thus prepared is available as a mixture of

pSTV29 and the above-described recombinant plasmid. When each DNA sample was subjected to a digestion with the AccIII restriction endonuclease, the pSTV29 in all samples exhibited resistance to the AccIII restriction endonuclease activity, demonstrating the insertion of the AccIII modification enzyme gene into the recombinant plasmid contained in the DNA sample. Furthermore, when the DNA sample was simultaneously cleaved with the restriction endonucleases HindIII and XbaI, followed by analysis of the length of the resulting DNA fragments by agarose gel electrophoresis, the presence of ORF2 in the recombinant plasmid was confirmed. ORF2 was thus proven to be the AccIII modification enzyme gene. The base sequence of the AccIII modification enzyme gene obtained and the amino acid sequence deduced therefrom are shown by SEQ ID NO:14 and SEQ ID NO:13 in the Sequence Listing, respectively.

The structure of the AccIII restriction modification system gene demonstrated according to the present invention is shown in Figure 16, wherein M, R and the arrow represent the modification enzyme gene, the restriction endonuclease gene, and the orientation of ORF, respectively.

The present invention provides a plasmid vector capable of introducing into a host an exogenous desired gene encoding a protein lethal or harmful to the host, and a method for being capable of efficiently expressing the 5 protein using the plasmid vector for the first time. A method for being capable of isolating a restriction endonuclease gene which constitutes a restriction modification system without co-existence of a modification enzyme gene, which has been difficult in the prior arts, 10 is also provided. Furthermore, an AccIII restriction endonuclease gene and an AccIII modification enzyme gene are isolated by the present invention, and, from *Escherichia coli* transformed with the plasmid containing the gene, it is possible to easily obtain an AccIII 15 restriction endonuclease or an AccIII modification enzyme available in the genetic engineering at a desired purity of an enzyme preparation compared to the prior method for purification.

SEQUENCE LISTING

SEQ ID NO:1

SEQUENCE LENGTH: 295

SEQUENCE TYPE: amino acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: peptide

FEATURE: 2 (Val or Leu)

SEQUENCE DESCRIPTION:

Met Xaa Pro Leu Asp Lys Asp Leu Gln Lys Ala Lys Ile Ser Ile  
1 5 10 15  
Thr Asp Phe Phe Glu Ile Thr Asn Arg Val Leu Asp Tyr Phe Pro  
20 25 30  
Asn Val Ile Asn Asn Thr Val Glu Lys Gly Asp Tyr Leu Ile Ser  
35 40 45  
Ser Ser Asn Ile Ala Gly Thr Ile Lys Phe Leu Arg Pro Ile Asn  
50 55 60  
Arg Lys Leu Phe Ile Gln Glu Lys Lys Val Phe Asn Asp Tyr Phe  
65 70 75  
Gln Lys Leu Ile Ile Val Phe Glu Asn Ile Arg Asn Lys Lys Thr  
80 85 90  
Val Thr Glu Glu Asp Lys Ile Ile Asp Arg Val Ile Tyr Thr  
95 100 105  
Ile Gln Gln Ser Ile Gly Ile Gly Leu Asp Leu Met Val Asn Gln  
110 115 120  
Asn Ser Ala Arg Lys His Val Gly Asn Arg Phe Glu Glu Leu Ile  
125 130 135  
Arg Val Ile Phe Thr Glu Ile Ser Val Ser Asn Lys Arg Thr Val  
140 145 150  
Leu Gln Ile Pro Tyr Glu Thr Asp Glu Gly Gln Lys Ile Tyr Lys  
155 160 165  
Cys Glu Asn Asp Leu Ile Ile Ser Pro Phe Glu Asn Val Glu Ser

170	175	180
Thr Asn Lys His Leu Asp Glu Asn Glu Ile Val Val Ser Ile Lys		
185	190	195
Thr Thr Ser Lys Asp Arg Met Gly Lys Met Phe Ile Asp Lys Ile		
200	205	210
Leu Leu Glu Arg Phe Val Lys His Pro Gln Lys Val Ile Gly Ile		
215	220	225
Phe Leu Asn Asp Val Gln Arg Lys Glu Asp Asn Asn Ile Ser Phe		
230	235	240
Thr Leu Val Ser Gly Leu Phe Met Val Tyr Thr Lys Phe Leu Thr		
245	250	255
Thr Leu Glu Gly Ile Tyr Tyr Leu Asp Pro Pro Pro Asn Ala Leu		
260	265	270
Lys Leu Pro Tyr Ser Asn His Met Lys Arg Phe Ser Asp Leu Ile		
275	280	285
Thr Glu Asp Leu Glu Lys Leu Phe Ser Ser		
290	295	

SEQ ID NO:2

SEQUENCE LENGTH: 885

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: Genomic DNA

SEQUENCE DESCRIPTION:

ATGSTACCACTGGATAAAAGA TTTACAAAAAA GCAAAGATTTCATTACTGA TTTTTTGAA 60  
ATTACAAATA GAGTTTACA TTATTTCCCC AATGTAATCA ATAATACAGT TGAAAAAGGA 120  
GATTATTTAA TATCCTCATC AAATATTGCT GGAACAATAA AATTCCCTAAG ACCAATCAAT 180  
AGAAAGTTAT TTATTCAGGA AAAAAAAGTT TTCAATGATT ATTTCAAAA ACTGATTATA 240  
GTTTTGAAA ATATAAGGAA CAAAAAAACT GTAACAGAGG AAGATAAAAT TATTATTGAT 300  
AGGGTAATTT ACACAATACA GCAATCTATT GGAATTGGTT TAGATTTAAT GGTTAACCAA 360  
AATAGTGCTA GAAAGCACGT TGGTAACCGA TTTGAAGAAT TAATTAGAGT CATTTCACAA 420  
GAAATATCAG TATCGAATAA AAGAACTGTA TTACAAATTC CATATGAAAC TGATGAAGGA 480  
CAGAAAATTT ACAAAATGCGA GAATGACCTC ATTATTTCTC CTTTGAAAAA TGTAGAATCT 540

ACAAACAAAC ATCTAGATGA AAATGAGATT GTTGGTTCAA TAAAGACAAC ATCAAAAGAT	600
AGGATGGAA AAATGTTAT AGATAAAATT TTACTTGAAA GGTTTGTAA ACACCCTCAA	660
AAAGTTATAG GGATTTCCCT CAATGATGTA CAAAGAAAAG AAGACAACAA TATCAGCTT	720
ACACTTGTTC CAGGATTATT TATGGTGTAT ACTAAATTCT TAACTACTCT TGAAGGGATC	780
TATTATTTAG ATCCACCACC TAATGCATTG AAACTACCAT ATTCTAATCA TATGAAAAGA	840
TTTCAGATT TAATTACAGA AGACCTTGAA AAATTATTCT CCTCT	885

SEQ ID NO:3

SEQUENCE LENGTH: 215

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

TATGGATATG TTCATAAACCA CGCATGTAGG CAGATAGATC TTTGGTTGTG AATCGCAACC	60
AGTGGCCTTA TGGCAGGAGC CGCGGATCAC CTACCATCCC TAATGACCTG CAGGCATGCA	120
AGCTTGCATG CCTGCAGGTC ATTAGGTACG GCAGGGTGTGC TCGAGGCGAA GGAGTGCCTG	180
CATGCGTTTC TCCTTGGCTT TTTCCCTCTG GGACA	215

SEQ ID NO:4

SEQUENCE LENGTH: 215

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

TATGTCCCAG AGGAAAAAAAG CCAAGGAGAA ACGCATGCAG GCACTCCTTC GCCTCGAGCA	60
CACCTGCCGT ACCTAATGAC CTGCAGGCAT GCAAGCTTGC ATGCCTGCAG GTCATTAGGG	120
ATGGTAGGTG ATCCGGGGCT CCTGCCATAA GGCCACTGGT TGCGATTAC AACCAAAGAT	180
CTATCTGCCT ACATGCGTGT TTATGAACAT ATCCA	215

SEQ ID NO:5

SEQUENCE LENGTH: 28

SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
MOLECULE TYPE: Other nucleic acid (synthetic DNA)  
SEQUENCE DESCRIPTION:  
AGATCTAGAG CAAACAAAAA AACCAACCG

28

SEQ ID NO:6  
SEQUENCE LENGTH: 24  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
MOLECULE TYPE: Other nucleic acid (synthetic DNA)  
SEQUENCE DESCRIPTION:  
GGTCTAGATC CCAGAGGAAA AAAG

24

SEQ ID NO:7  
SEQUENCE LENGTH: 100  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
MOLECULE TYPE: Other nucleic acid (synthetic DNA)  
SEQUENCE DESCRIPTION:  
CTCGAGATT AGGTGACACT ATAGAATACG GAATTGTGAG CGGATAACAA TTCCAAGCTT      60  
CACAGGAAAC AGACCATGGC TTAAGTAACT AGTGAATTCG                                100

SEQ ID NO:8  
SEQUENCE LENGTH: 100  
SEQUENCE TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
MOLECULE TYPE: Other nucleic acid (synthetic DNA)  
SEQUENCE DESCRIPTION:  
CGAATTCACT AGTTACTTAA GCCATGGTCT GTTCCTGTG AAGCTTGGAA TTGTTATCCG      60

CTCACAAATTC CGTATTCTAT AGTGTCACCT AAATCTCGAG

100

SEQ ID NO:9

SEQUENCE LENGTH: 27

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

AATCCCATGG AACGCTACGA ATCTCTG

27

SEQ ID NO:10

SEQUENCE LENGTH: 29

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

CCGGCCATGG TTATTTTGACCCAGACC

29

SEQ ID NO:11

SEQUENCE LENGTH: 26

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

TAACTTGAAT CCATGGGTTC TCACCG

26

SEQ ID NO:12

SEQUENCE LENGTH: 29

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

TACTCAGTAG CCATGGCTCT CATAGACCG

29

SEQ ID NO:13

SEQUENCE LENGTH: 308

SEQUENCE TYPE: amino acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE DESCRIPTION:

Met	Asn	Glu	Ile	Ala	Phe	Asp	Asn	Tyr	Ser	Tyr	Ile	Pro	Lys	Leu	
1															15
Lys	Leu	Tyr	Ser	Glu	Ile	Glu	Leu	Lys	Pro	Phe	Phe	Ile	Ser	Lys	
															30
Asn	Gly	Ser	Leu	Phe	Asn	Val	Asp	Ala	Ile	Asp	Phe	Leu	Arg	Lys	
															45
Leu	Glu	Ser	Asn	Ser	Val	Asp	Leu	Ile	Phe	Ala	Asp	Pro	Pro	Tyr	
															60
Asn	Ile	Lys	Lys	Ala	Glu	Trp	Asp	Ile	Phe	Ser	Ser	Gln	Asn	Glu	
															75
Tyr	Leu	Glu	Trp	Ser	Lys	Glu	Trp	Ile	Met	Glu	Ala	His	Arg	Val	
															90
Leu	Lys	Asp	Asn	Gly	Ser	Leu	Tyr	Val	Cys	Gly	Phe	Ser	Glu	Ile	
															105
Leu	Ala	Asp	Ile	Lys	Phe	Ile	Thr	Ser	Lys	Tyr	Phe	His	Ser	Cys	
															120
Lys	Trp	Leu	Ile	Trp	Phe	Tyr	Arg	Asn	Lys	Ala	Asn	Leu	Gly	Lys	
															135
Asp	Trp	Gly	Arg	Ser	His	Glu	Ser	Ile	Leu	Leu	Leu	Arg	Lys	Ser	
															150
Lys	Asn	Phe	Ile	Phe	Asn	Ile	Asp	Glu	Ala	Arg	Ile	Pro	Tyr	Asn	
															165
Glu	His	Thr	Val	Lys	Tyr	Pro	Gln	Arg	Thr	Gln	Ala	Glu	Ser	Ser	

170	175	180
Gln Tyr Ser Asn Ser Lys Lys Gln Tyr Ile Trp Glu Pro Asn Pro		
185	190	195
Leu Gly Ala Lys Pro Lys Asp Val Leu Glu Ile Pro Thr Ile Ser		
200	205	210
Asn Gly Ser Trp Glu Arg Ser Ile His Pro Thr Gln Lys Pro Val		
215	220	225
Glu Leu Leu Lys Lys Ile Ile Leu Ser Ser Ser Asn Lys Asp Ser		
230	235	240
Leu Ile Leu Asp Pro Phe Gly Gly Ser Gly Thr Thr Tyr Ala Val		
245	250	255
Ala Glu Ala Phe Gly Arg Lys Trp Ile Gly Thr Glu Leu Asp Lys		
260	265	270
Asn Tyr Cys Leu Glu Ile Gln Lys Arg Leu Lys Asp Glu Ser Met		
275	280	285
Ile Asn Arg Ile Phe Ser Gly Asp Asp Asp Ser Asn Ser Gln Asn		
290	295	300
Arg Arg Lys Lys Leu Arg Gly Glu		
305		

SEQ ID NO:14

SEQUENCE LENGTH: 924

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: Genomic DNA

SEQUENCE DESCRIPTION:

GTGAATGAAA TAGCGTTGA TAATTACAGT TATATACCAA AATTAAACT TTATTCCGAA 60  
ATCGAGCTTA AACCAATTAA TATTCAAAA AACGGTTCAC TTTCAATGT TGATGCTATT 120  
GATTTTTAA GAAAATTAGA GAGTAATTCT GTGGATTAA TTTTGAGA TCCACCTTAT 180  
AACATTAAAA AGGCAGAGTG GGATATTTT TCTTCTCAA ATGAATATCT CGAATGGAGT 240  
AAAGAATGGA TAATGGAAGC TCATAGAGTT TTAAAAGATA ATGCCAGTTT ATATGTTGT 300  
GGCTTTCAAG AAATTCTGGC AGACATAAAA TTTATCACTT CAAAATATT TCACAGTTGT 360  
AAATGGTTGA TTTGGTTCTA TAGAAACAAG GCAAATTTAG GTAAAGATTG GGGACGTTCA 420

CACGAAAGTA TACTGTTATT AAGAAAATCT AAAAATTTA TTTTAATAT TGATGAGGCA 480  
CGAATCCCGT ATAATGAGCA TACAGTTAAA TATCCACAAA GAACCCAGGC CGAATCTTCG 540  
CAATATTCGA ACTCAAAAAA GCAATATATT TGGGAGCCAA ACCCATTAGG AGCTAAGCCA 600  
AAAGATGTTT TGGAGATTCC CACAATTCA AATGGTTCTT GGGAAAGAAG TATTCACCCCT 660  
ACGCAAAAGC CAGTAGAATT GCTAAAAAA ATAATTTAT CTTCATCTAA TAAAGATAGT 720  
TTAATTCTTG ATCCATTGG TGGTCGGGA ACTACATATG CTGTTGCGGA AGCTTTGGC 780  
AGAAAATGGA TTGGAACAGA GTTAGATAAA AATTATTGTC TGGAAATTCA AAAGCGATTG 840  
AAAGACGAAA GTATGATCAA CAGGATTTT TCAGGCGATG ATGATTCAA TTCTCAAAAT 900  
AGAAGAAAAA AATTAAGAGG AGAA 924

SEQ ID NO:15

SEQUENCE LENGTH: 29

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

TCGAGATT TA GGTGACACTA TAGAATACA

29

SEQ ID NO:16

SEQUENCE LENGTH: 29

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

AGCTTGTATT CTATAGTGTC ACCTAAATC

29

SEQ ID NO:17

SEQUENCE LENGTH: 54

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

- 90 -

SEQUENCE DESCRIPTION:

TCGAGATTAGGTGACACTATAGAATACGG AATTGTGAGCGGATAACAAT TCCA

54

SEQ ID NO:18

SEQUENCE LENGTH: 54

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

AGCTTGGAAAT TGTTATCCGC TCACAATTCC GTATTCTATA GTGTCACCTA AATC

54

SEQ ID NO:19

SEQUENCE LENGTH: 20

SEQUENCE TYPE: amino acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: peptide

SEQUENCE DESCRIPTION:

Met Leu Pro Leu Asp Lys Asp Leu Gln Lys Ala Lys Ile Ser Ile

1

5

10

15

Thr Asp Phe Phe Glu

20

SEQ ID NO:20

SEQUENCE LENGTH: 23

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

FEATURE: 6, 9, 12 (inosine)

SEQUENCE DESCRIPTION:

ATGTTNCCNY TNGAYAARGA YYT

23

SEQ ID NO:21

SEQUENCE LENGTH: 23

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

FEATURE: 9 (inosine)

SEQUENCE DESCRIPTION:

AAGGATTTCNC ARAARGCNAARAT

23

SEQ ID NO:22

SEQUENCE LENGTH: 30

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

TAAATCTAAA CCAATTCCAA TAGATTGCTG

30

SEQ ID NO:23

SEQUENCE LENGTH: 30

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

CAAATCGGTT ACCAACGTGC TTTCTAGCAC

30

SEQ ID NO:24

SEQUENCE LENGTH: 30

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

GAACTGTATT ACAAATTCCA TATGAAACTG

30

SEQ ID NO:25

SEQUENCE LENGTH: 30

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

GACAGAAAAT TTACAAATGC GAGAATGACC

30

SEQ ID NO:26

SEQUENCE LENGTH: 1588

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: Genomic DNA

SEQUENCE DESCRIPTION:

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AGACATCCTG CATATGACGG TGGATGAAGC ATTGGAATTT TTCTCGGAAA ATCACGAAGA 120  
AAAAATTGTA ACCAAACTAA AACCTTGCA GGACGTTGGT TTGGGTTATC TTCAGTTAGG 180  
CCAGTCCTCC TCTACTCTT CCGGCGGTGA AGCCCAAAGA GTGAAGCTCG CCTCTTCCT 240  
TGTGAAAGGT GTAACGACGG AAAAAACGTT ATTTGTTTT GATGAACCAT CAACAGGATT 300  
ACATTTCCAC GACATTCAAA AATTACTGAA ATCACTTCAG GCACTGATAG AATTAGGGCA 360  
TTCGGTTGTA GTGATTGAGC ATCAGCCGGA TATTATCAA TGCGCCGATT ACATCATCGA 420  
TGTCGGACCC AATGCCGGA AATACGGTGG CGAAATTGTT TTCACAGGAA CTCCGGAAAGA 480  
TTTGGTAAAA GAGAAAAAGT CGTTTACAGG GAAGTATATT AAGGAGAAGT TAAAGTAATT 540  
TATTTATATT TGAAGTTATG CTACCACTGG ATAAAGATT ACAAAAGCA AAGATTCAA 600  
TTACTGATTT TTTGAAATT ACAAAATAGAG TTTTAGATTA TTTCCCCAAT GTAATCAATA 660  
ATACAGTTGA AAAAGGAGAT TATTTAATAT CCTCATCAA TATTGCTGGA ACAATAAAAT 720  
TCCTAAGACC AATCAATAGA AAGTTATTTA TTCAGGAAAA AAAAGTTTC AATGATTATT 780  
TTCAAAAAC GATTATAGTT TTTGAAAATA TAAGGAACAA AAAAACTGTA ACAGAGGAAG 840  
ATAAAATTAT TATTGATAGG GTAATTACA CAATACAGCA ATCTATTGGA ATTGGTTAG 900

ATTTAATGGT TAATCAAAAT AGTGCTAGAA AGCACGTTGG TAACCGATT GAAGAATTAA 960  
TTAGAGTCAT TTTTACAGAA ATATCAGTAT CGAATAAAAG AACTGTATTA CAAATTCCAT 1020  
ATGAAACTGA TGAAGGACAG AAAATTACA AATGCGAGAA TGACCTCATT ATTTCTCCTT 1080  
TTGAAAATGT AGAATCTACA AACAAACATC TAGATGAAAA TGAGATTGTT GTTTCAATAA 1140  
AGACAAACATC AAAAGATAGG ATGGGAAAAA TGTTTATAGA TAAAATTAA CTTGAAAGGT 1200  
TTGTTAAACA CCCTCAAAAA GTTATAGGGA TTTCTCAA TGATGTACAA AGAAAAGAAG 1260  
ACAACAATAT CAGCTTACA CTTGTTCAAG GATTATTTAT GGTGTATACT AAATTCTTAA 1320  
CTACTCTGA AGGGATCTAT TATTTAGATC CACCACCTAA TGCATTGAAA CTACCATATT 1380  
CTAACATCAT GAAAAGATT TCAGATTAA TTACAGAAGA CCTTGAAAAA TTATTCTCCT 1440  
CTTAATTTT TTCTTCTATT TTGAGAATT GAATCATCAT CGCCTGAAAA AATCCTGTTG 1500  
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GTTCCAATCC ATTTCTGCC AAAAGCTT 1588

SEQ ID NO:27

SEQUENCE LENGTH: 25

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

ATATTTGAAG CCATGGTACC ACTGG 25

SEQ ID NO:28

SEQUENCE LENGTH: 26

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Oyther nucleic acid (synthetic DNA)

SEQUENCE DESCRIPTION:

GATGATTCAA ATTCTCACCA TGGAAAG 26

SEQ ID NO:29

SEQUENCE LENGTH: 4146

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: Genomic DNA

SEQUENCE DESCRIPTION:

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TCACAAGCCT TATCATCAC AC TCACAAAAGA GCAGAACAG TTCCTTGGA AAGGCGATAA 180  
AACCAGAAGT TTCCAAGTA TTGATAATT TTTCAAAATG CTTGAAGAGA ATCTTTACAA 240  
GATCCAATAC CGCGTAATGC TTTCGCGCTA TCGTGGGAAA ACACCTTGCC CCGATTGCGA 300  
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CCACGACAGA GAAATTGCCA AACGCCTGAC TTACGAAATC GAAACGAGAT TAGAATTCCCT 480  
GACGAAAGTC GGCCTTGGAT ATCTGACTTT GAACCGAACAA TCCAACACGC TTTCCGGAGG 540  
AGAAAGCCAG AGAATCAATC TGGCGACAGC TTGGGAAGTT CGCTGGTTGG TTCTATTAT 600  
ATTTGGATG AGCCGAGCAT TGGTCTGCAT TCCCCGCGATA CAGAAAATCT GATTGGTGTC 660  
CTCAAACAAAC TCCCGCGATTT GGGAAANTACC GTGATTGTTG TAGAACACGA CGAACATGTG 720  
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CTTGTTCAG GCGNGGATTA TAAAGAGATG CTGAAGTNTN ATACTTTAAC CGCAAATAC 840  
CTGAATGGCG AACTGAAAAT AGAAGTTCTT GAAAAACGAA GAAAACCGAA GGAATTCATC 900  
GCAATAAAAG GTGCCCGCCA GAATAATTAA AAAAATATTG ACGTTGATGT TCCGTTAGAA 960  
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GGTGGCGAAA TTGTTTCAC AGGAACCTCG GAGAATTTGG TAAAAGAGAA AAAGTCGTTT 1860  
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TCTTTATTG ATAAATAATC ATCCATATCT TACACAACAT TACACAAGTT TATACAGCAA 3900  
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SEQ ID NO:30

SEQUENCE LENGTH: 23

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: Genomic DNA

SEQUENCE DESCRIPTION:

ATTTAGGTGA CACTATAGAA TAC

23

CLAIMS

1. A plasmid vector characterized by comprising a promoter sequence to control an expression of a desired gene, said promoter sequence being recognized by an RNA polymerase not inherent to a host, and a replication origin for increasing a copy number by induction with an exogenous factor.
- 10 2. The plasmid vector according to claim 1, wherein said promoter sequence is recognized by RNA polymerases derived from bacteriophages.
- 15 3. The plasmid vector according to claim 2, wherein said promoter sequence is recognized by an RNA polymerase derived from SP6 phage.
- 20 4. The plasmid vector according to claim 3, wherein said promoter sequence contains the base sequence of SEQ ID NO:30 set forth in the Sequence Listing.
- 25 5. The plasmid vector according to any one of claims 1 to 4, wherein said replication origin is under control of a promoter.

6. The plasmid vector according to any one of claims 1 to 5, wherein said replication origin is under control of the lac promoter.

5 7. The plasmid vector according to any one of claims 1 to 6, comprising a drug resistance gene as a selection marker.

8. The plasmid vector according to claim 7, which is  
10 selected from pACE601, pACE611, pACE701 and pACE702.

9. A plasmid vector in which a desired gene to be expressed is incorporated into the plasmid vector according to any one of claims 1 to 8.

15 10. A method for expressing a desired gene, characterized by introducing into a host a plasmid vector in which the desired gene is incorporated into the plasmid vector according to any one of claims 1 to 8, and an RNA polymerase gene which recognizes a promoter sequence in said plasmid vector, and inducing an increase in a copy number of said plasmid vector and an expression of said RNA polymerase by using an exogenous factor to transcribe and translate the desired gene.

25

11. The method for expressing a desired gene according to claim 10, characterized in that the increase in the copy number of the plasmid vector and the expression of the RNA polymerase are induced by respective exogenous factors.

5

12. The method for expressing a desired gene according to claim 10, characterized in that the increase in the copy number of the plasmid vector and the expression of the RNA polymerase are induced by a same exogenous factor.

10

13. The method for expressing a desired gene according to any one of claims 10 to 12, wherein said exogenous factor which induces the increase in the copy number of the plasmid vector, is one or more selected from the group consisting of an addition of isopropyl- $\beta$ -D-thiogalactoside (IPTG), an addition of lactose, an addition of galactose, an addition of arabinose, a reduction of a tryptophane concentration and an adjustment of a transformant cultivation temperature.

15  
20

14. The method for expressing the desired gene according to any one of claims 10 to 12, wherein said exogenous factor which induces the expression of the RNA polymerase, is one or more selected from the group consisting of an addition of isopropyl- $\beta$ -D-thiogalactoside (IPTG), an

25

addition of lactose, an addition of galactose, an addition of arabinose, a reduction of a tryptophane concentration and an adjustment of a transformant cultivation temperature.

5

15. The method for expressing a desired gene according to claim 10, characterized in that said RNA polymerase gene is introduced into the host by the other plasmid vector or a phage vector.

10

16. The method for expressing a desired gene according to claim 10, characterized in that said RNA polymerase gene is incorporated into a chromosome of the host.

15

17. The method for expressing a desired gene according to claim 15 or 16, characterized in that said RNA polymerase gene is derived from SP6 phage.

20

18. The method for expressing a desired gene according to any one of claims 10 to 17, wherein said desired gene encodes a protein lethal or harmful to the host.

25

19. The method for expressing a desired gene according to any one of claims 10 to 18, characterized in that *Escherichia coli* is used as the host.

20. A method for isolating a desired gene, characterized in that the plasmid vector according to any one of claims 1 to 8 is employed in the method for isolating the desired gene.

5

21. The method for isolating a desired gene according to claim 20, wherein said desired gene encodes a protein lethal or harmful to a host.

10 22. The method for isolating a desired gene according to claim 21, wherein the gene encoding a protein lethal or harmful to the host is a restriction endonuclease gene.

15 23. A polypeptide containing the entire or a portion of the amino acid sequence shown by SEQ ID NO:1 set forth in the Sequence Listing, and possessing an activity of AccIII restriction endonuclease.

20 24. A polypeptide having an amino acid sequence resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:1 set forth in the Sequence Listing or a portion thereof, and possessing an activity of AccIII restriction endonuclease.

25

25. A DNA encoding a polypeptide which contains the entire or a portion of the amino acid sequence shown by SEQ ID NO:1 set forth in the Sequence Listing, and possesses an activity of AccIII restriction endonuclease.

5

26. A DNA containing the entire or a portion of the DNA shown by SEQ ID NO:2 set forth in the Sequence Listing wherein an expression product of said DNA possesses an activity of AccIII restriction endonuclease.

10

27. A DNA encoding a polypeptide resulting from at least one of deletion, addition, insertion or substitution of one or more amino acid residues in the amino acid sequence of SEQ ID NO:1 set forth in the Sequence Listing or a portion thereof, and possessing an activity of AccIII restriction endonuclease.

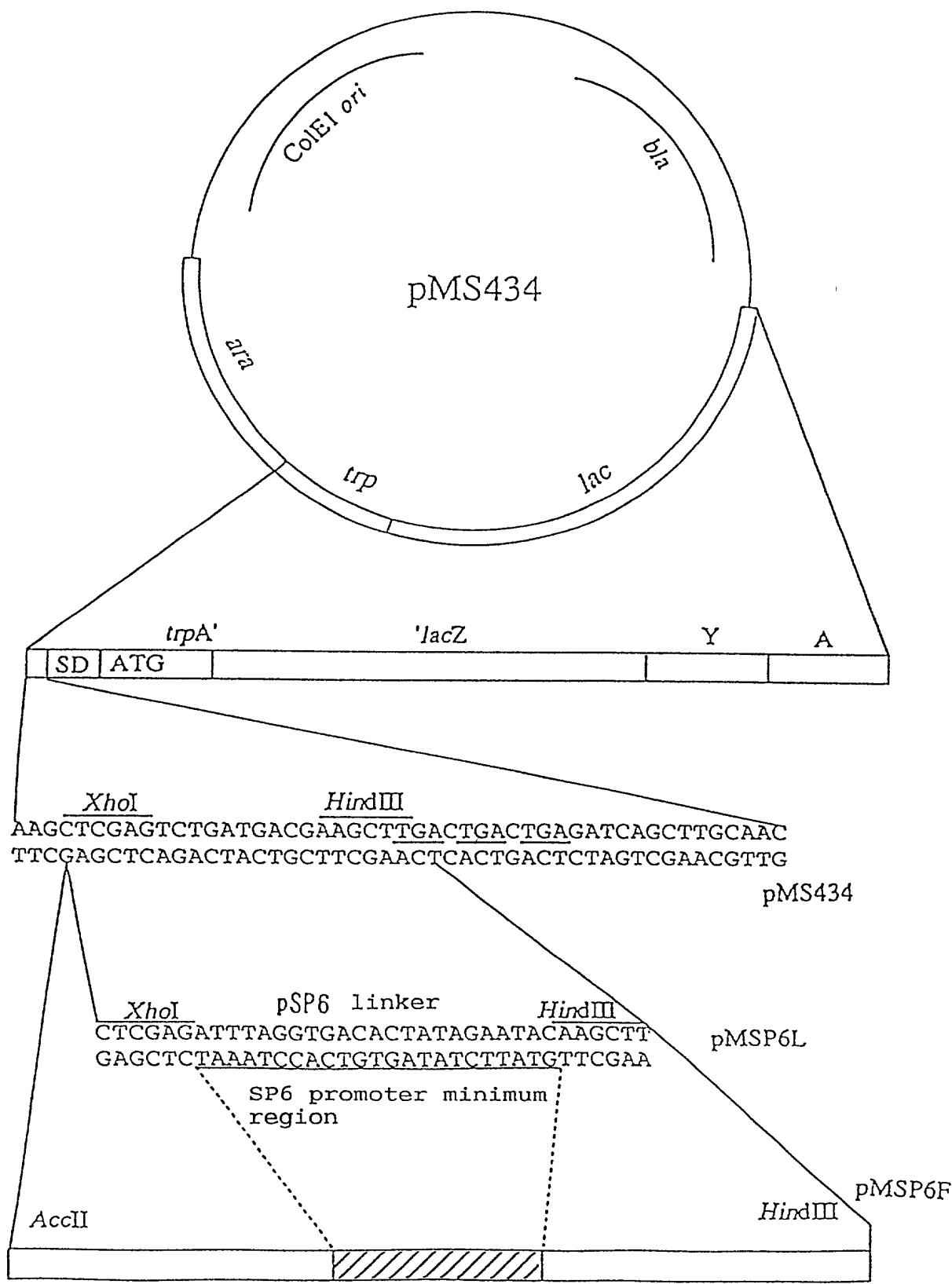
15

28. A DNA capable of hybridizing to the DNA according to any one of claims 25 to 27, and encoding a polypeptide possessing an activity of AccIII restriction endonuclease.

20

ABSTRACT

A plasmid vector characterized by comprising a promoter sequence that can be recognized by an RNA polymerase which is not inherent in a host and that controls the expression of desired genes and a replication origin that increases the number of copies under the induction by exogenous factors; methods for expression and isolation of target genes by using the vector; a polypeptide having the activity of an AccIII restriction endonuclease; and a DNA encoding the polypeptide. The invention provides for the first time a plasmid vector which can introduce an exogenous desired gene encoding proteins which are lethal or harmful to hosts into the hosts, a method for efficiently expressing the proteins by using the vector, and also a method for permitting a restriction endonuclease gene constituting a restriction-modification system to be isolated even in the absence of a modification enzyme gene, which has been difficult in the prior arts.



AccII-HindIII fragment from pSP64

FIG. 1

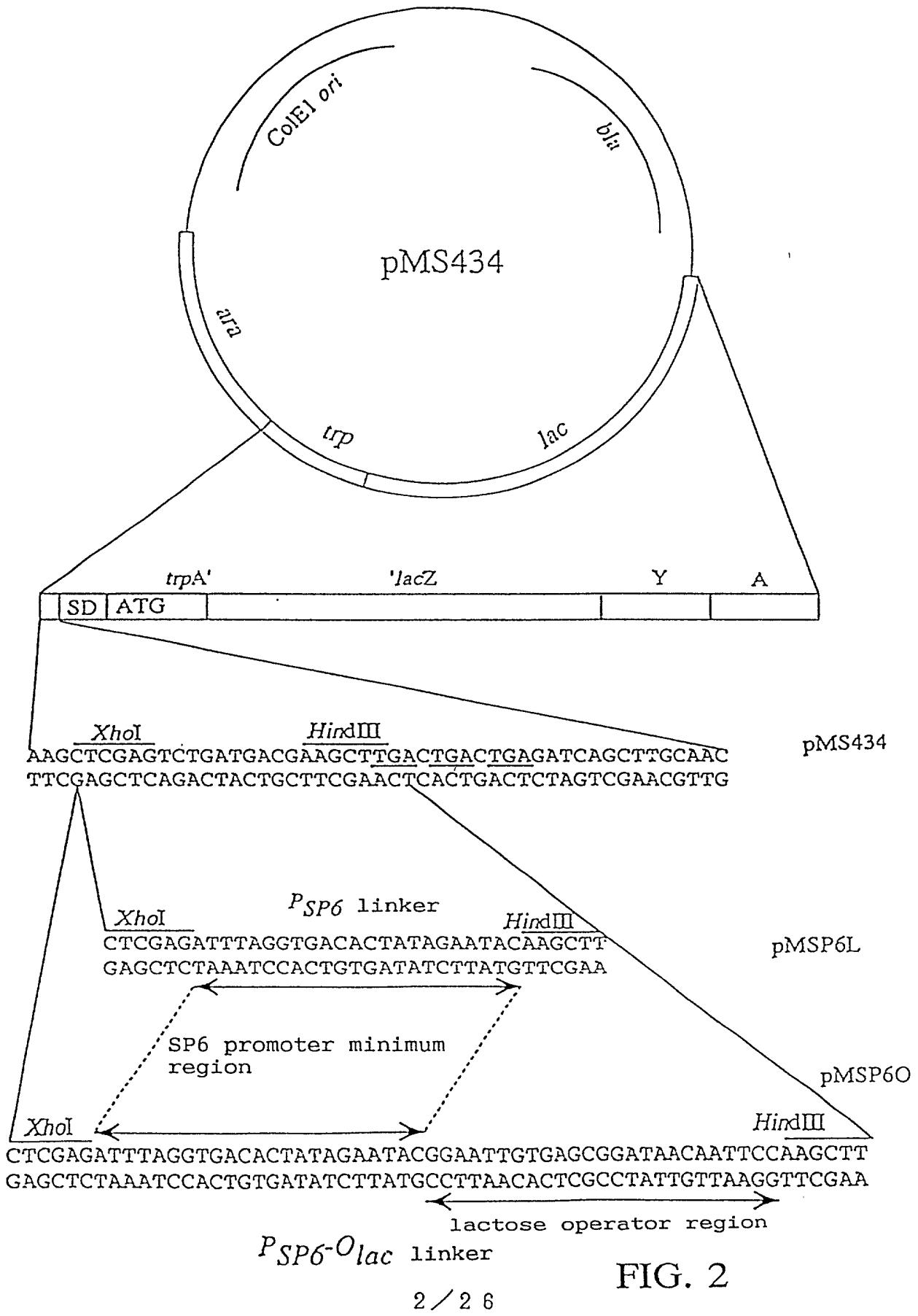
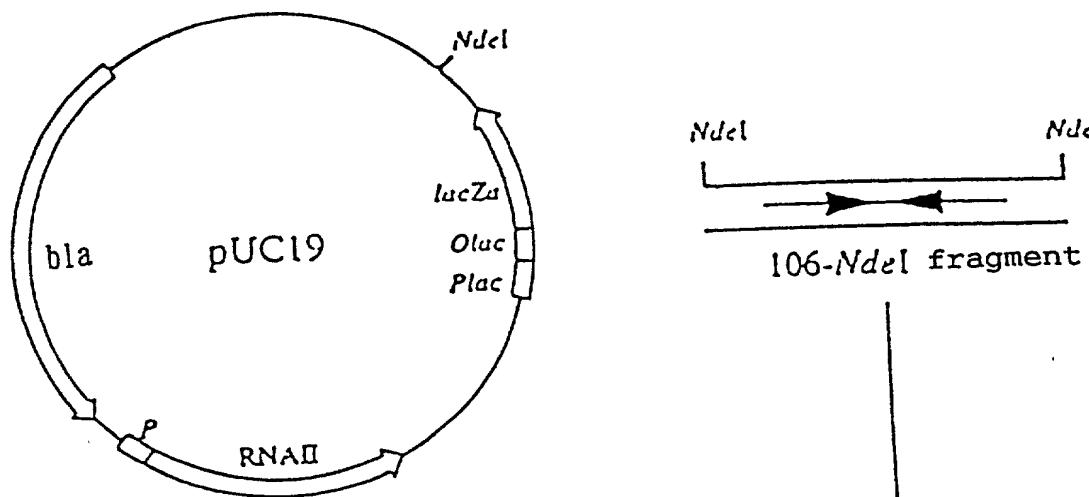
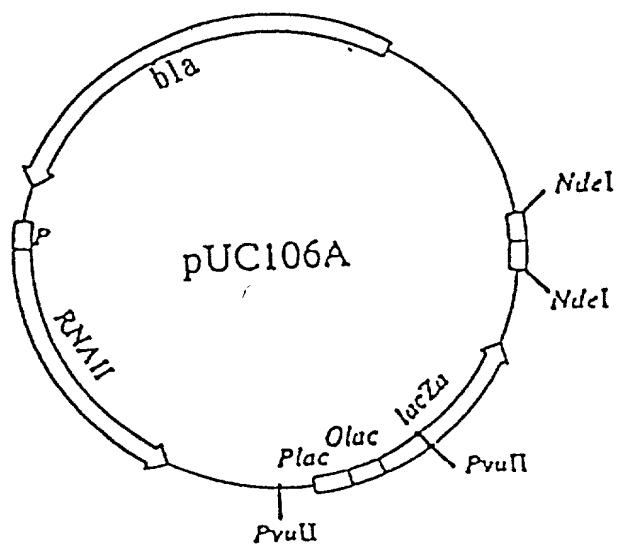


FIG. 2



**NdeI digestion**



**PvuII digestion**

**intramolecular ligation**

**pUC106AdPO**

**FIG. 3 A**

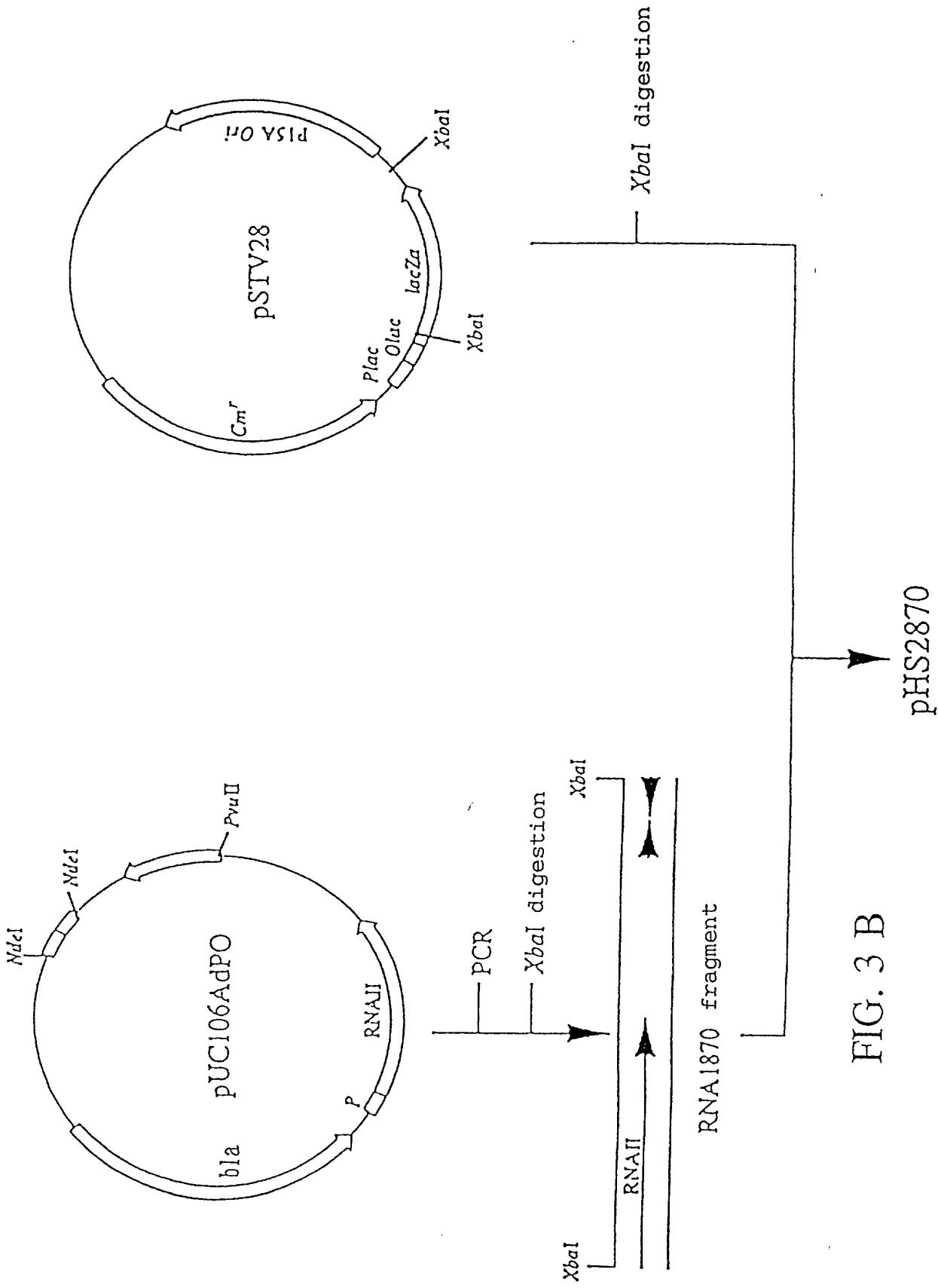


FIG. 3 B

pHS2870

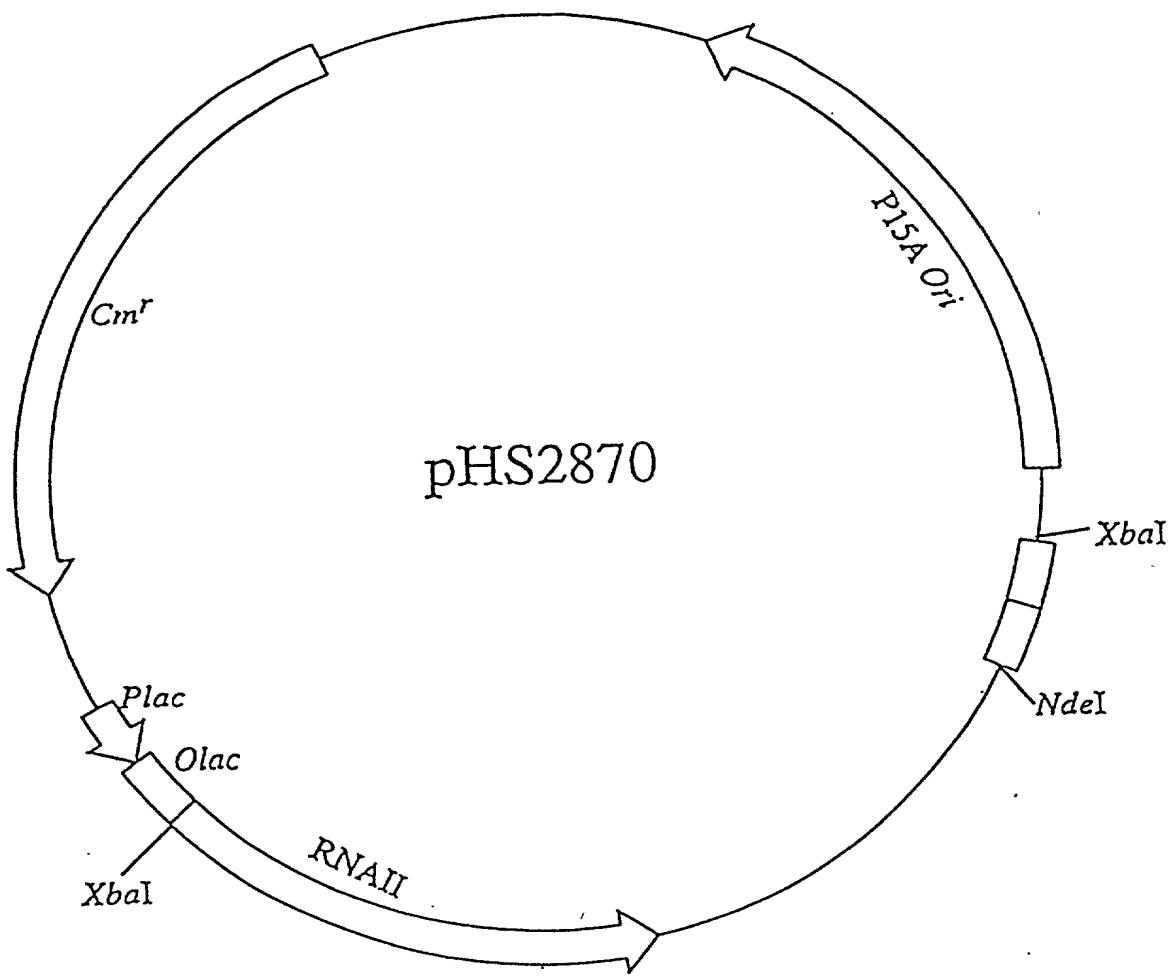


FIG. 4

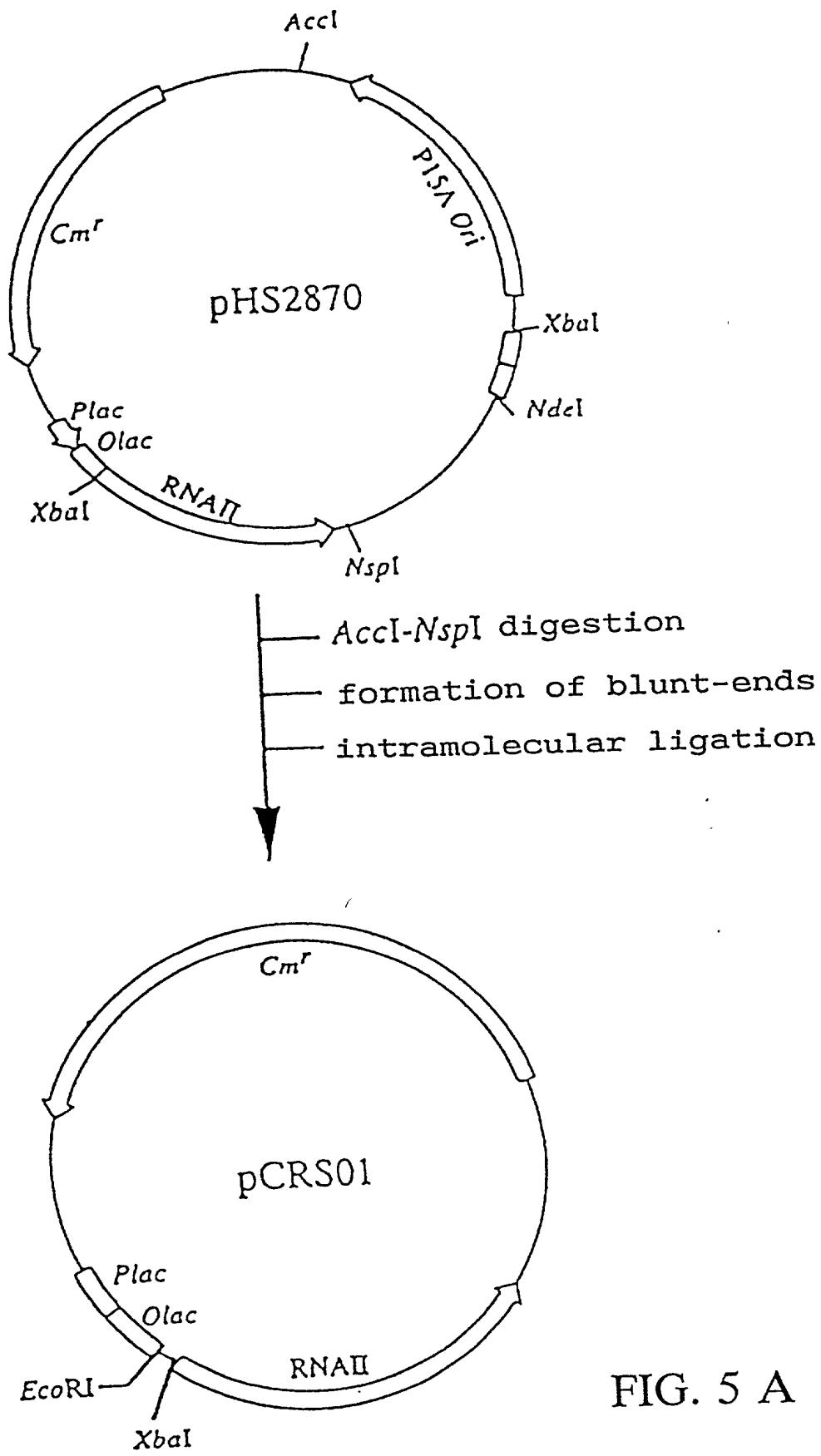


FIG. 5 A

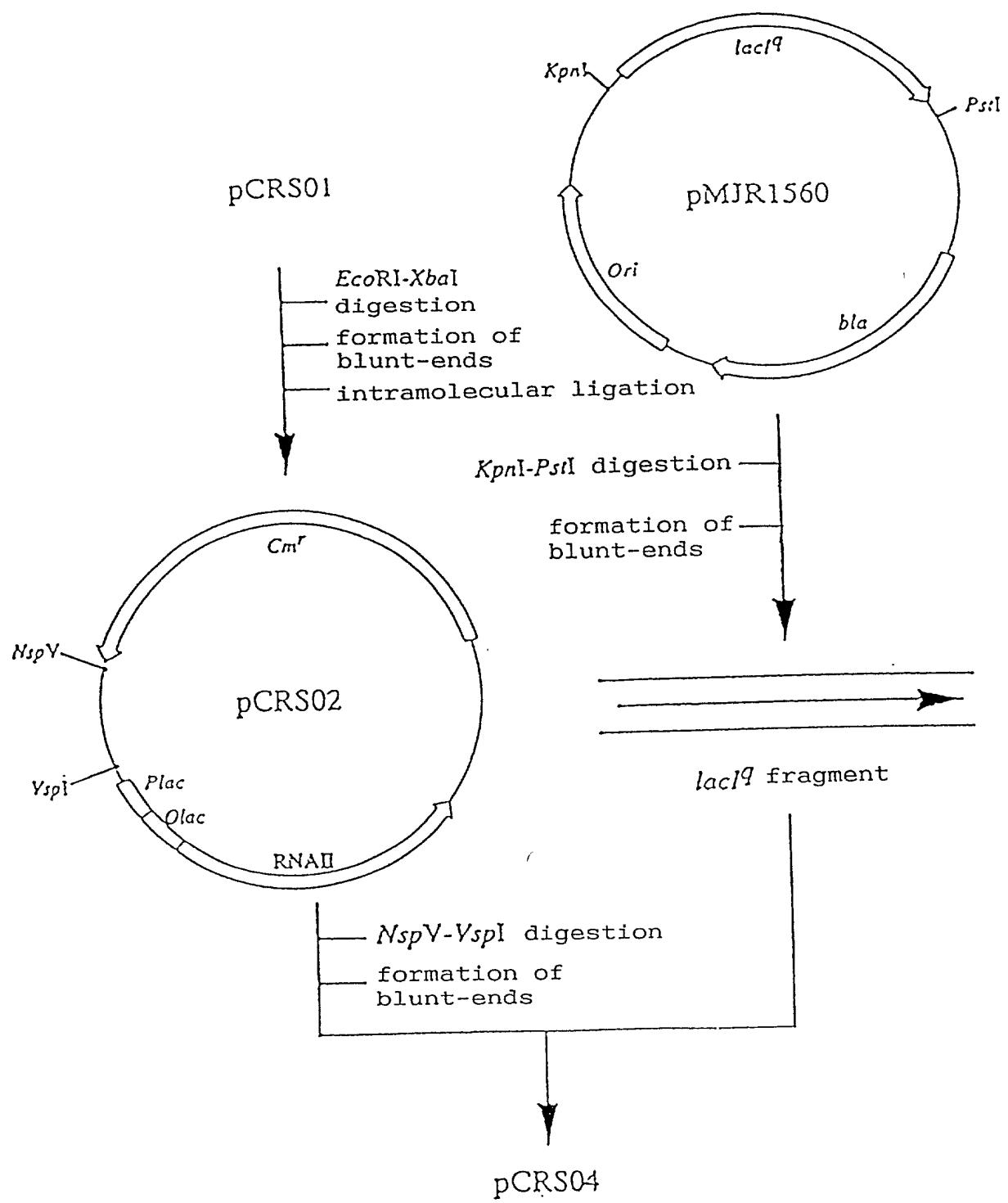


FIG. 5 B

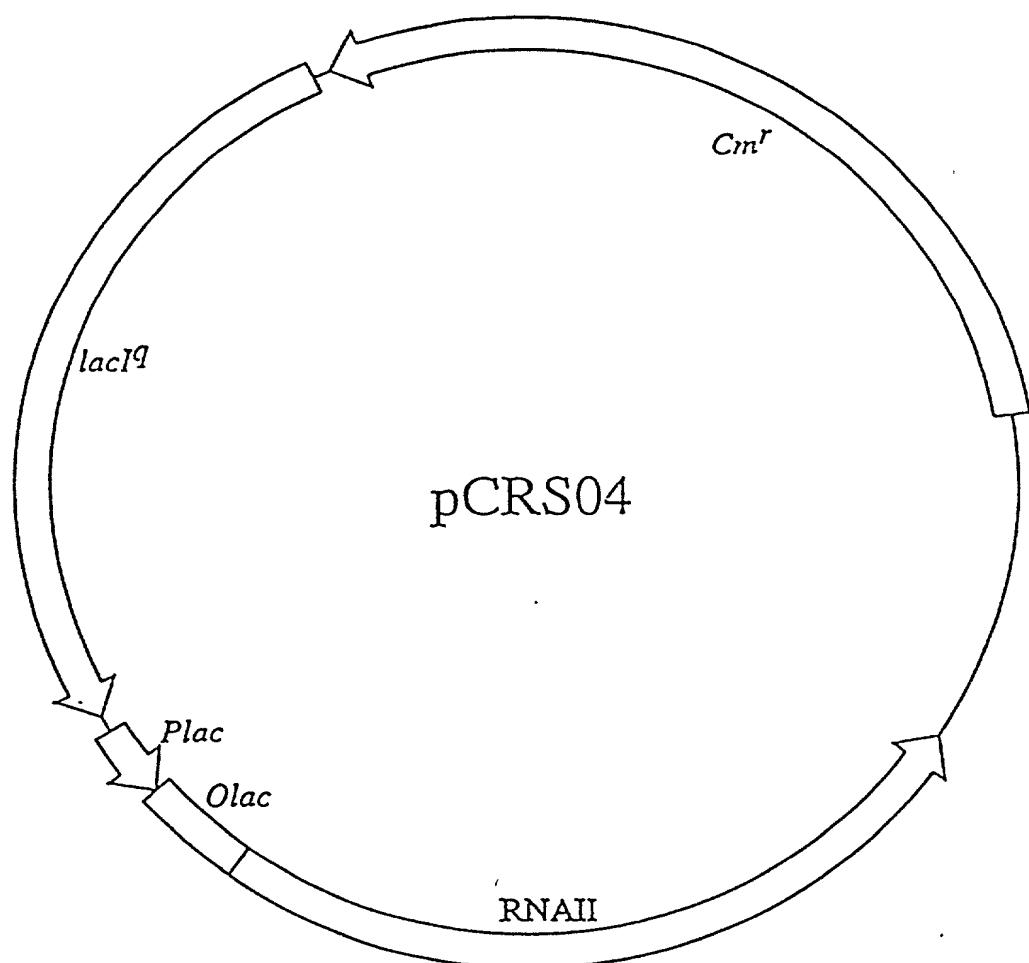


FIG. 6

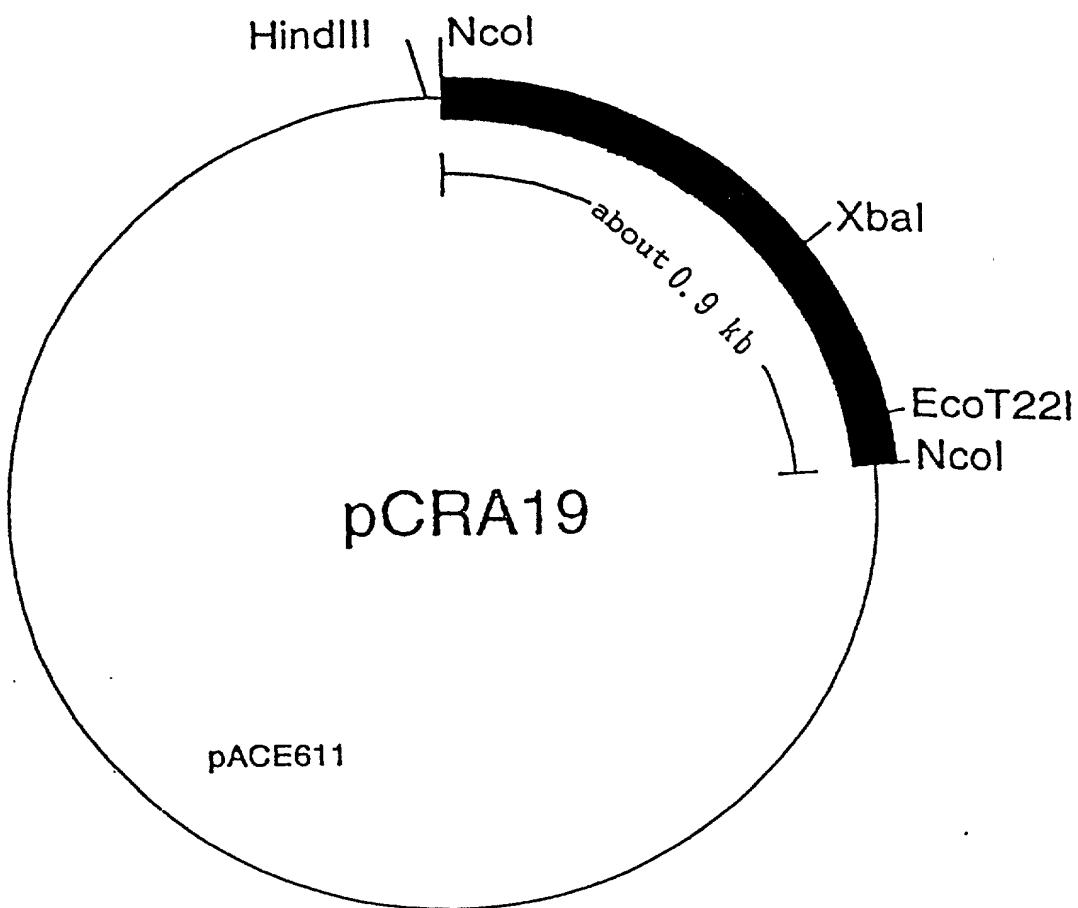


FIG. 7

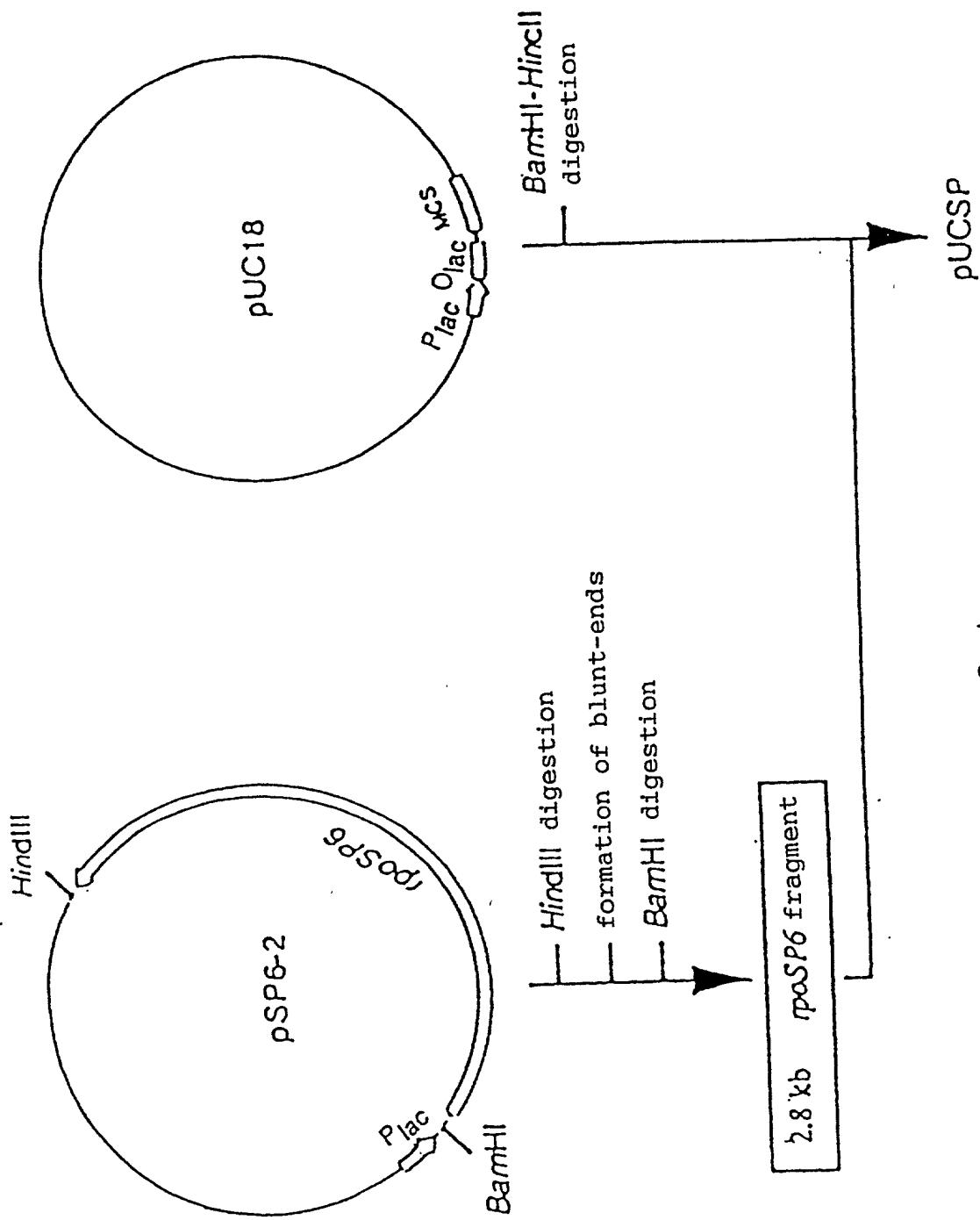


FIG. 8 A

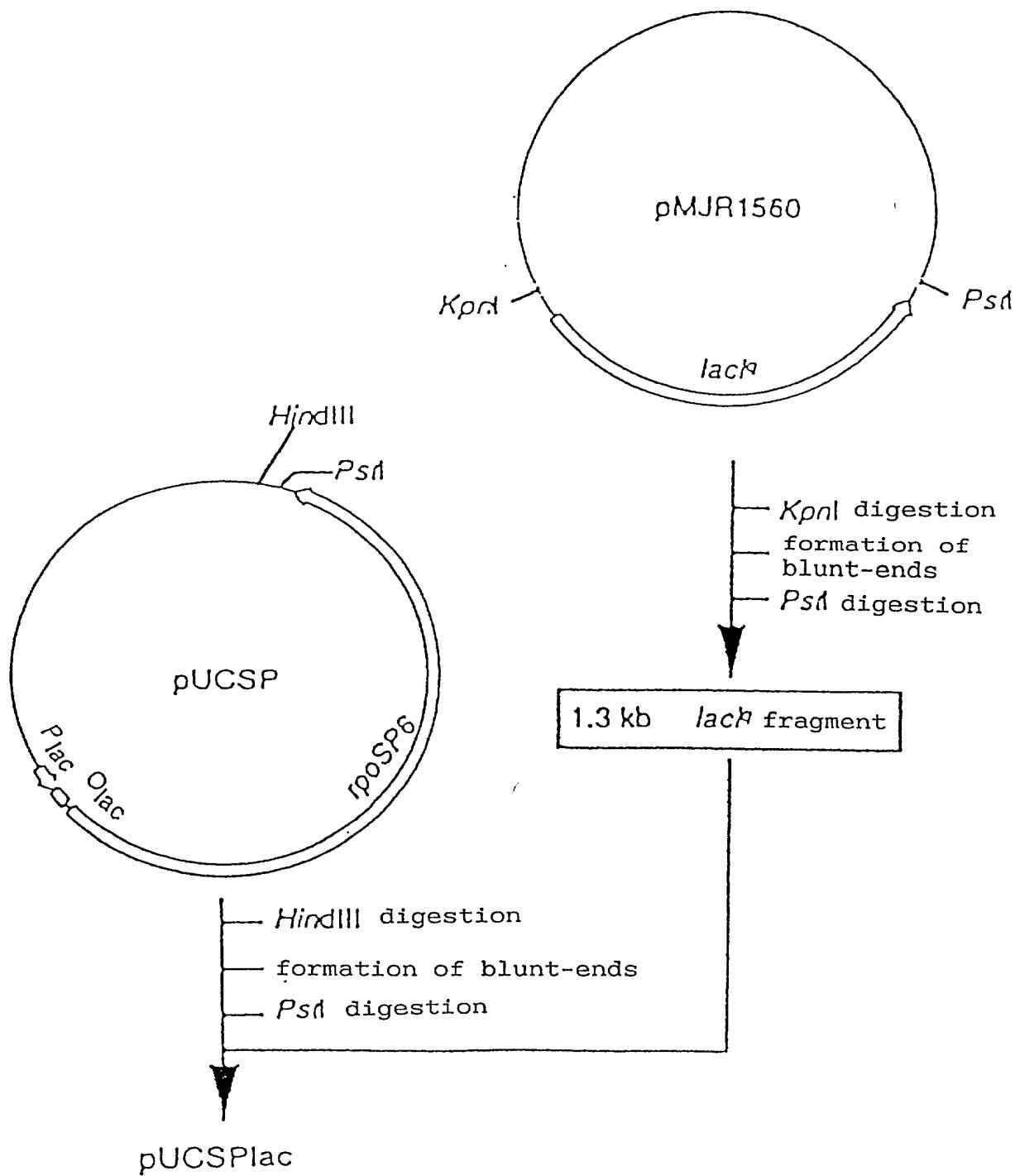


FIG. 8 B

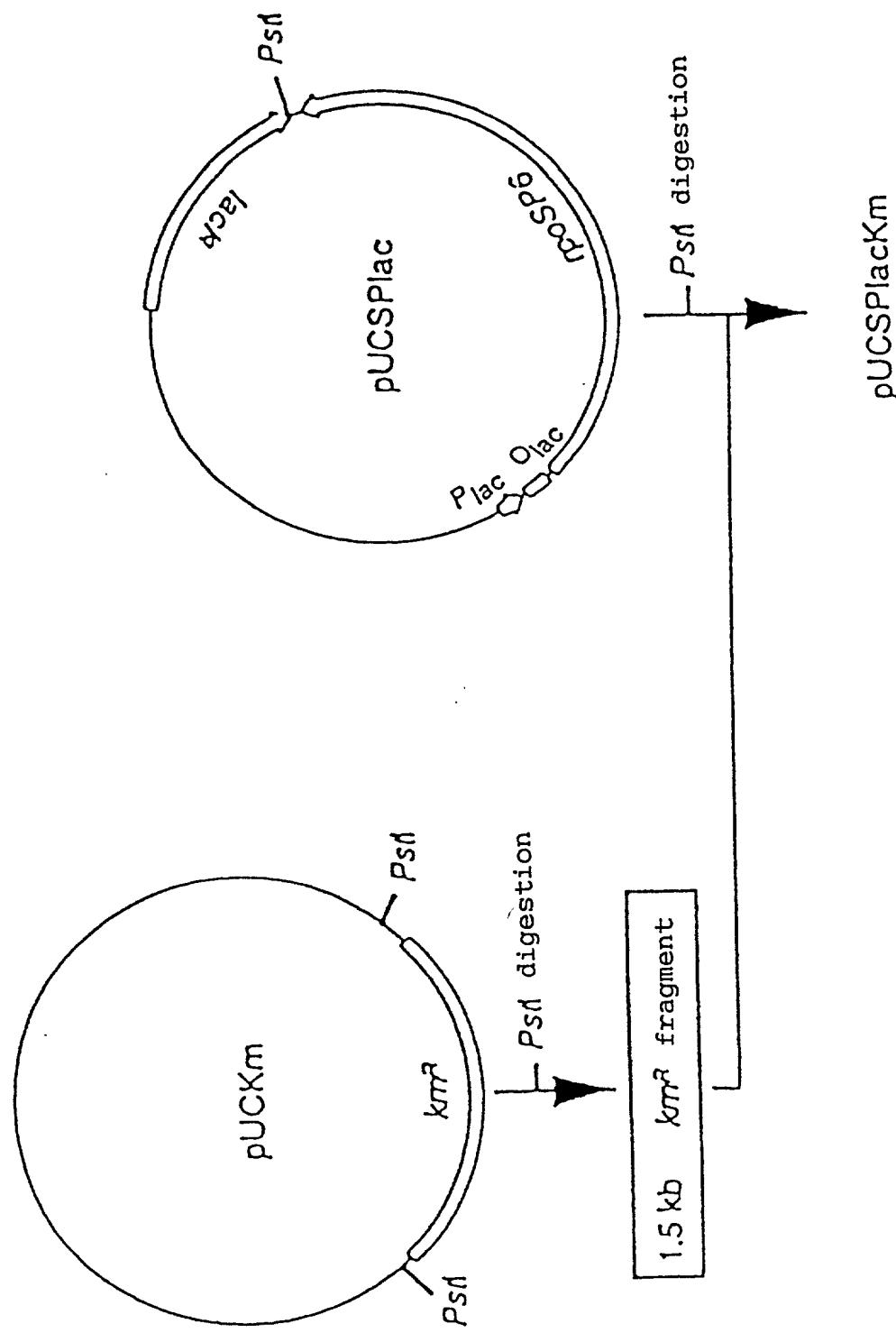


FIG. 8 C

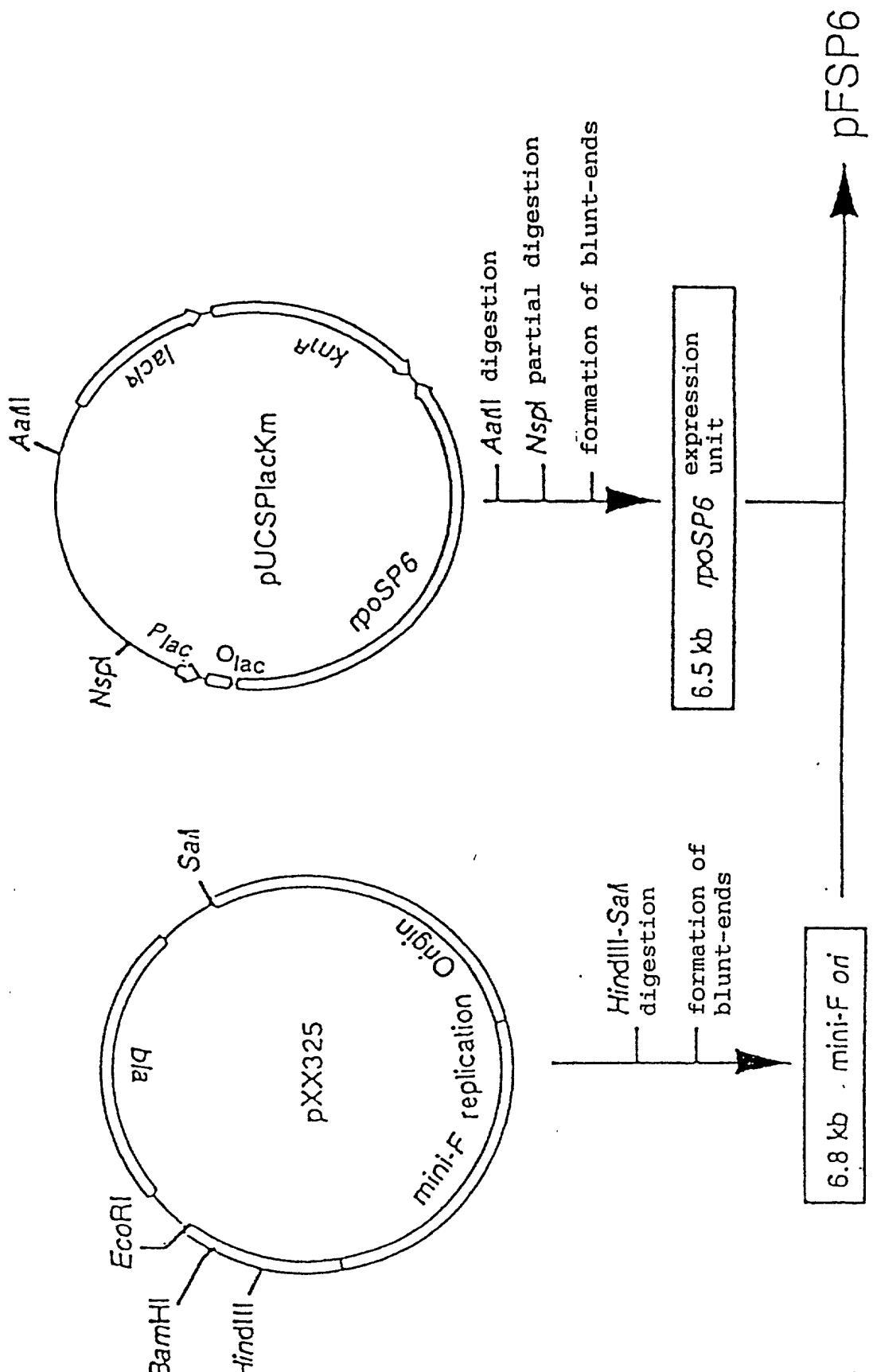


FIG. 8 D

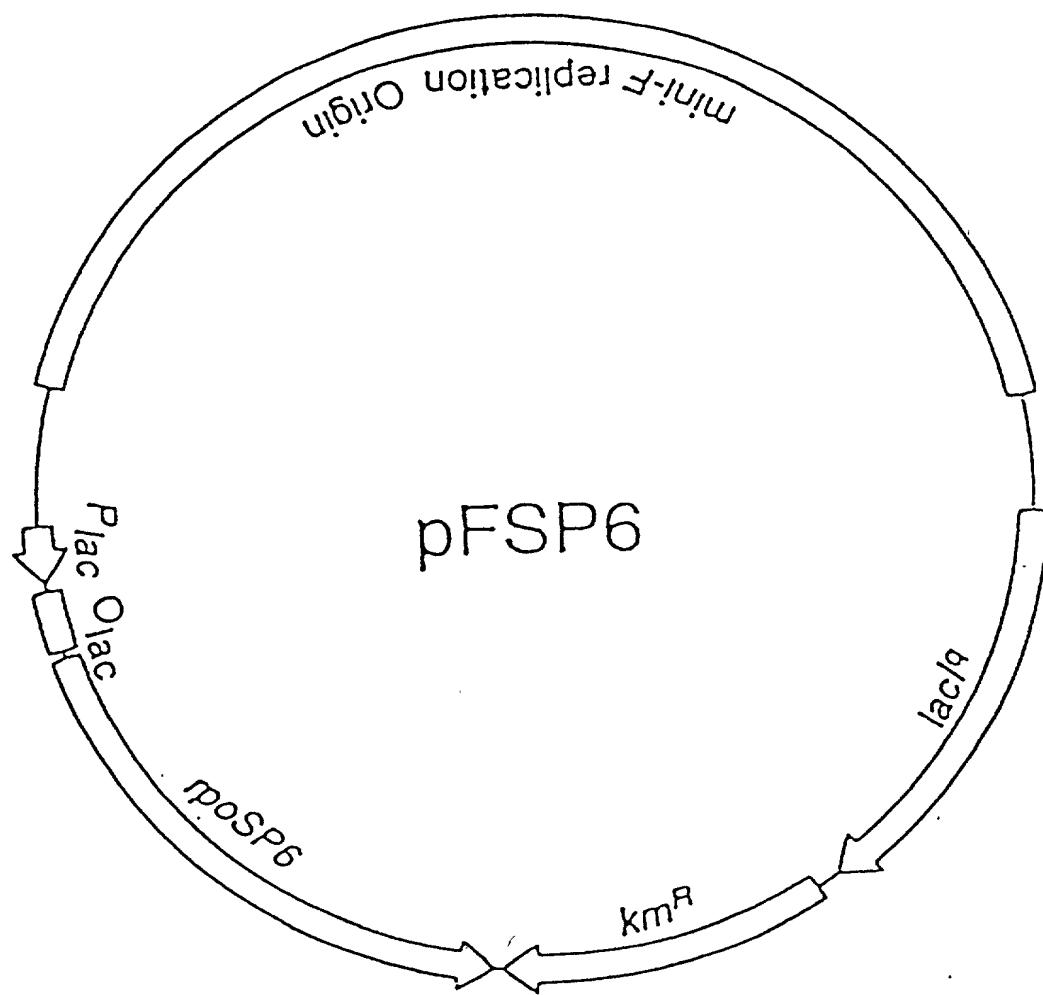


FIG. 8 E

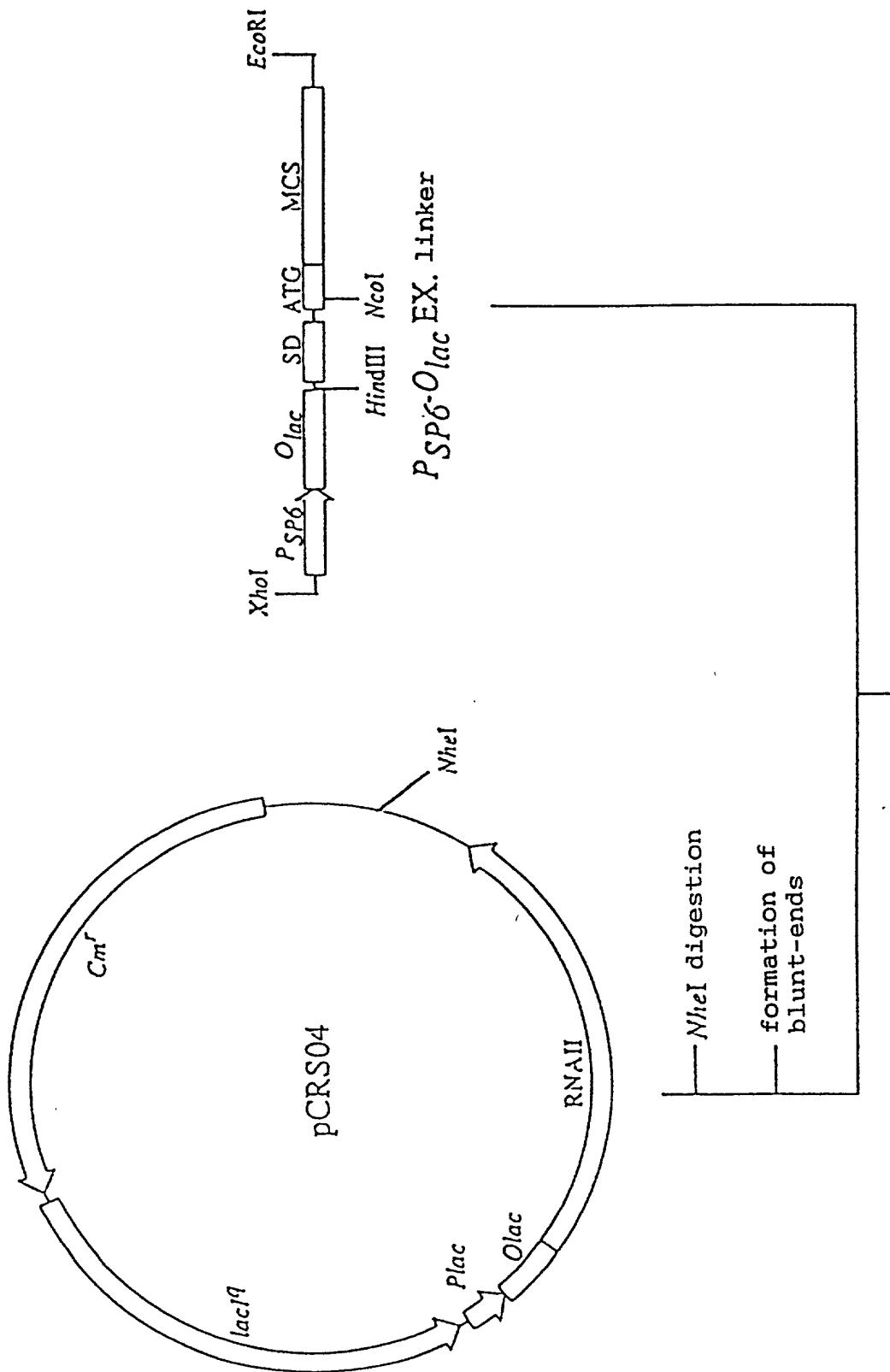


FIG. 9 A

PACE601

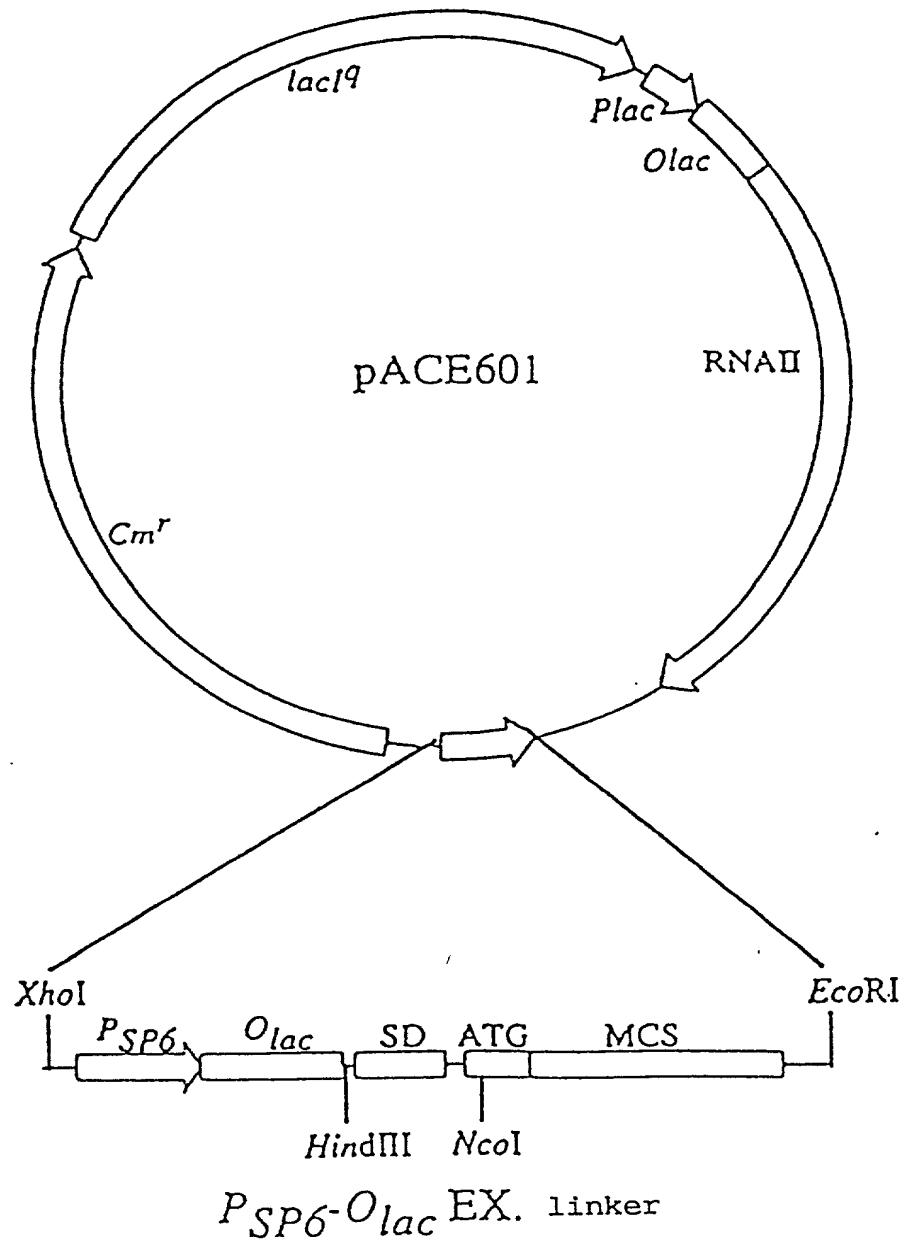


FIG. 9 B

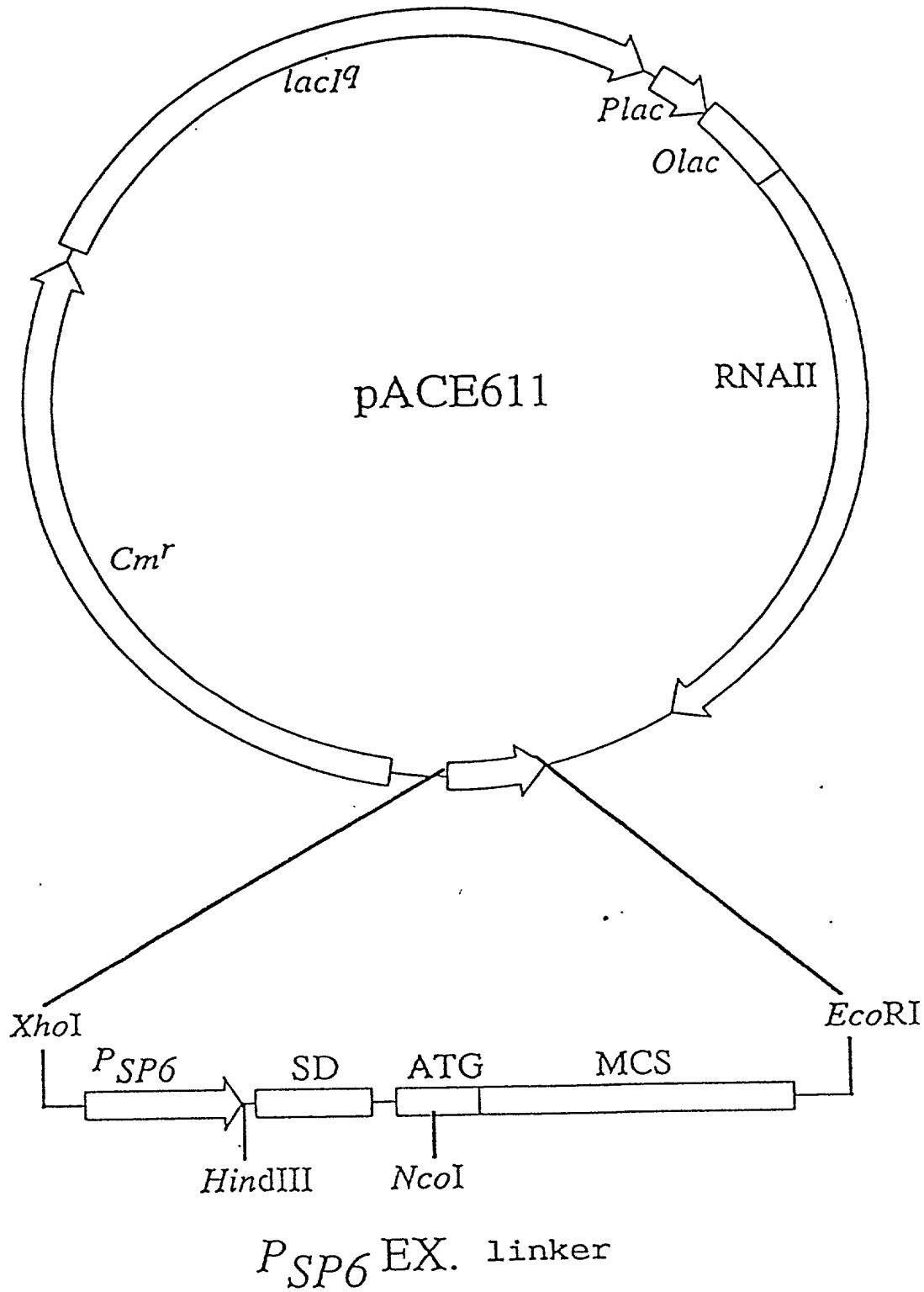


FIG. 10

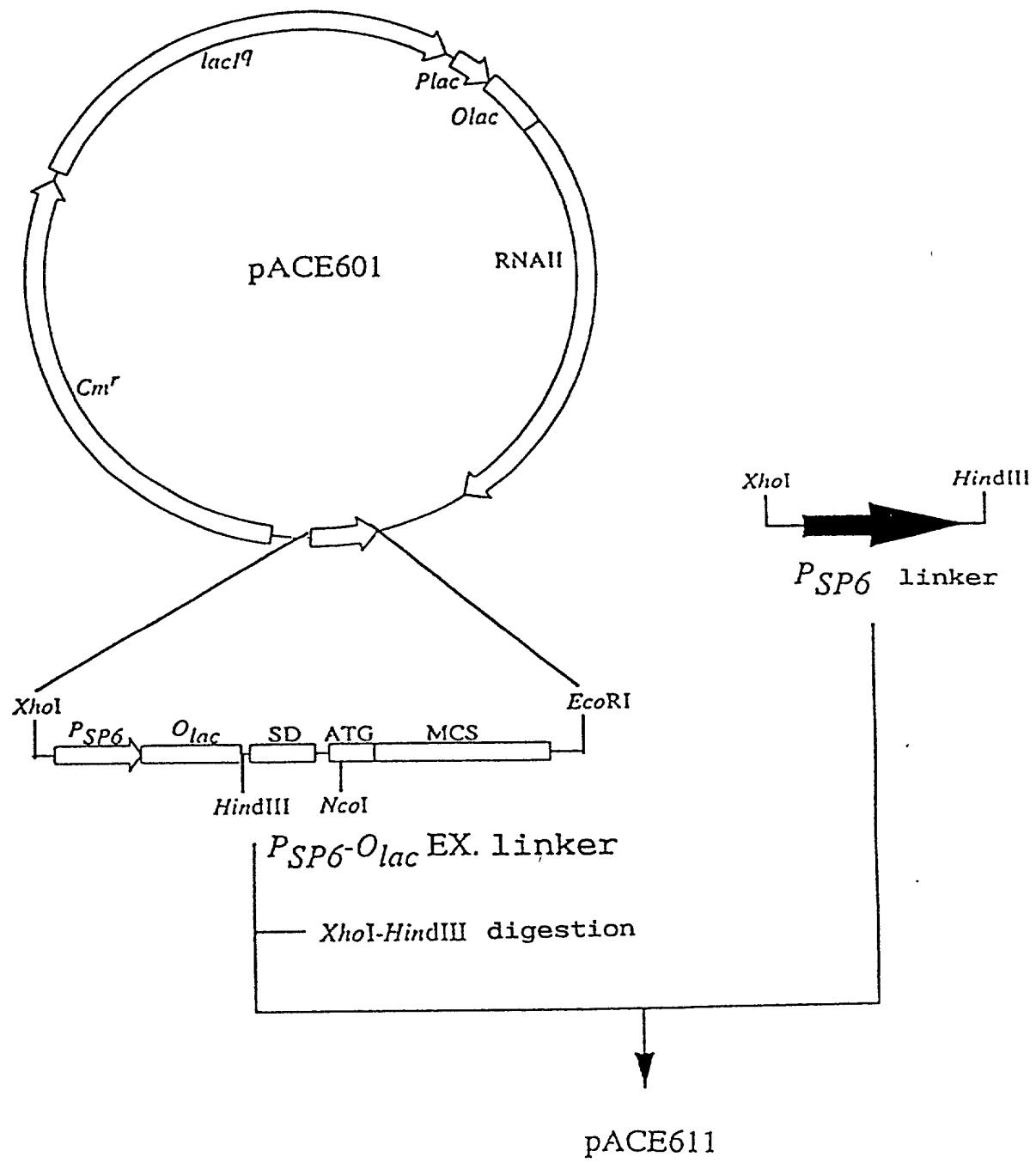


FIG. 11 A

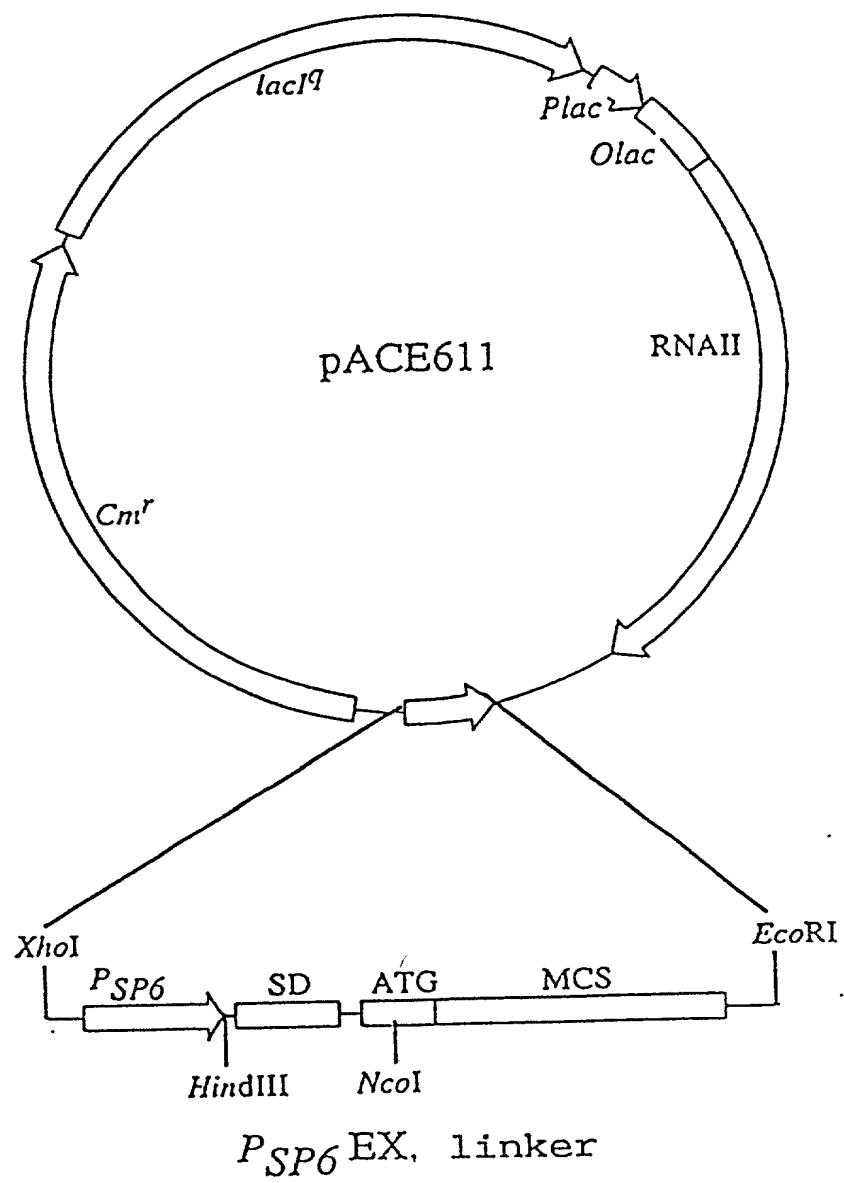


FIG. 11 B

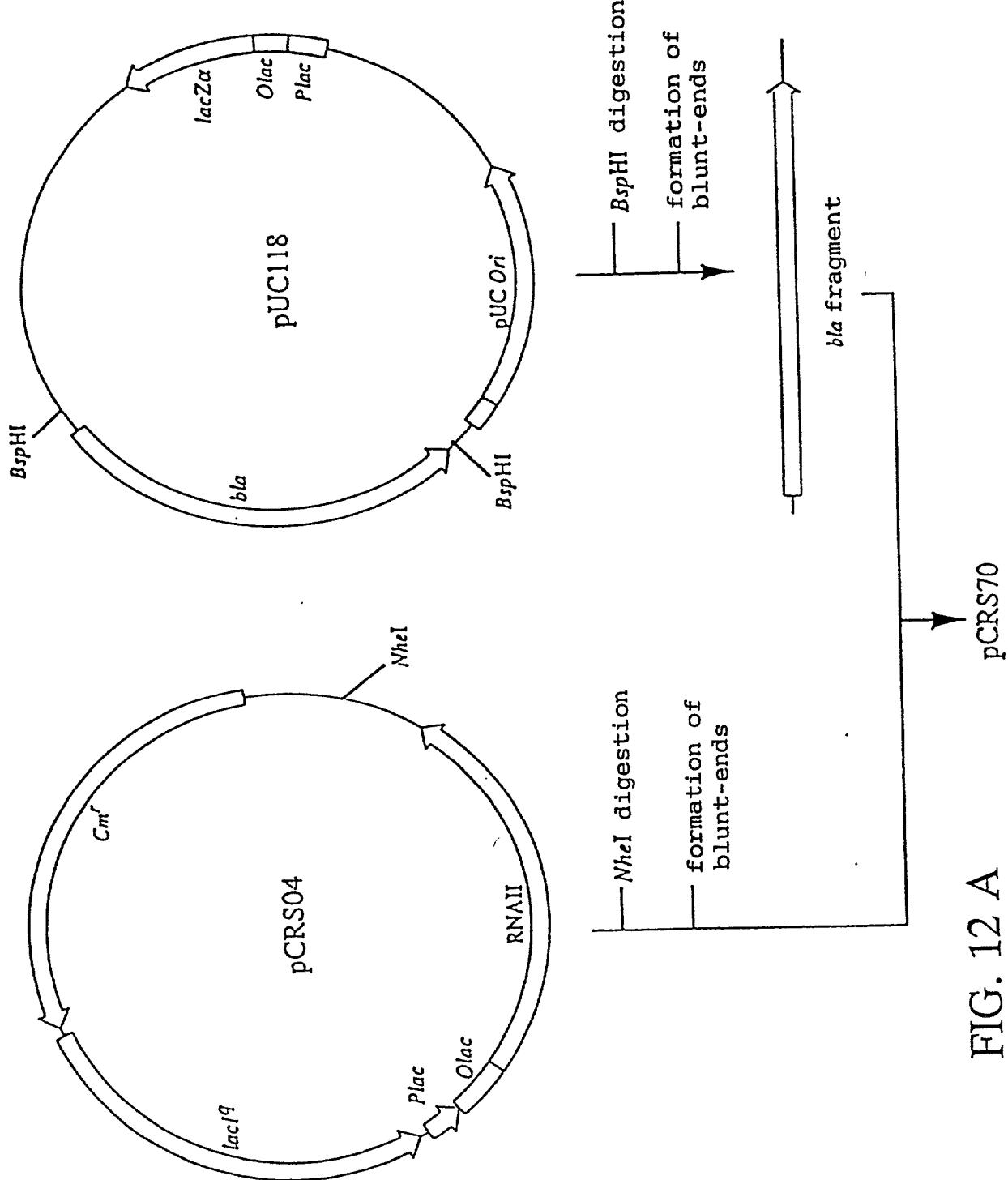


FIG. 12 A

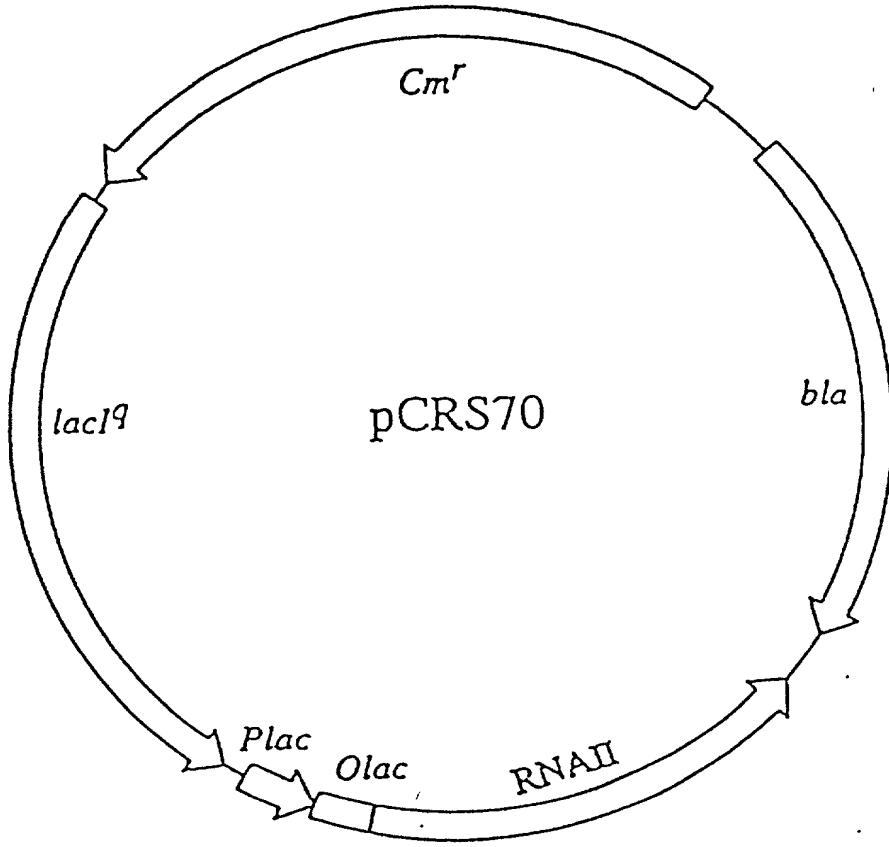


FIG. 12 B

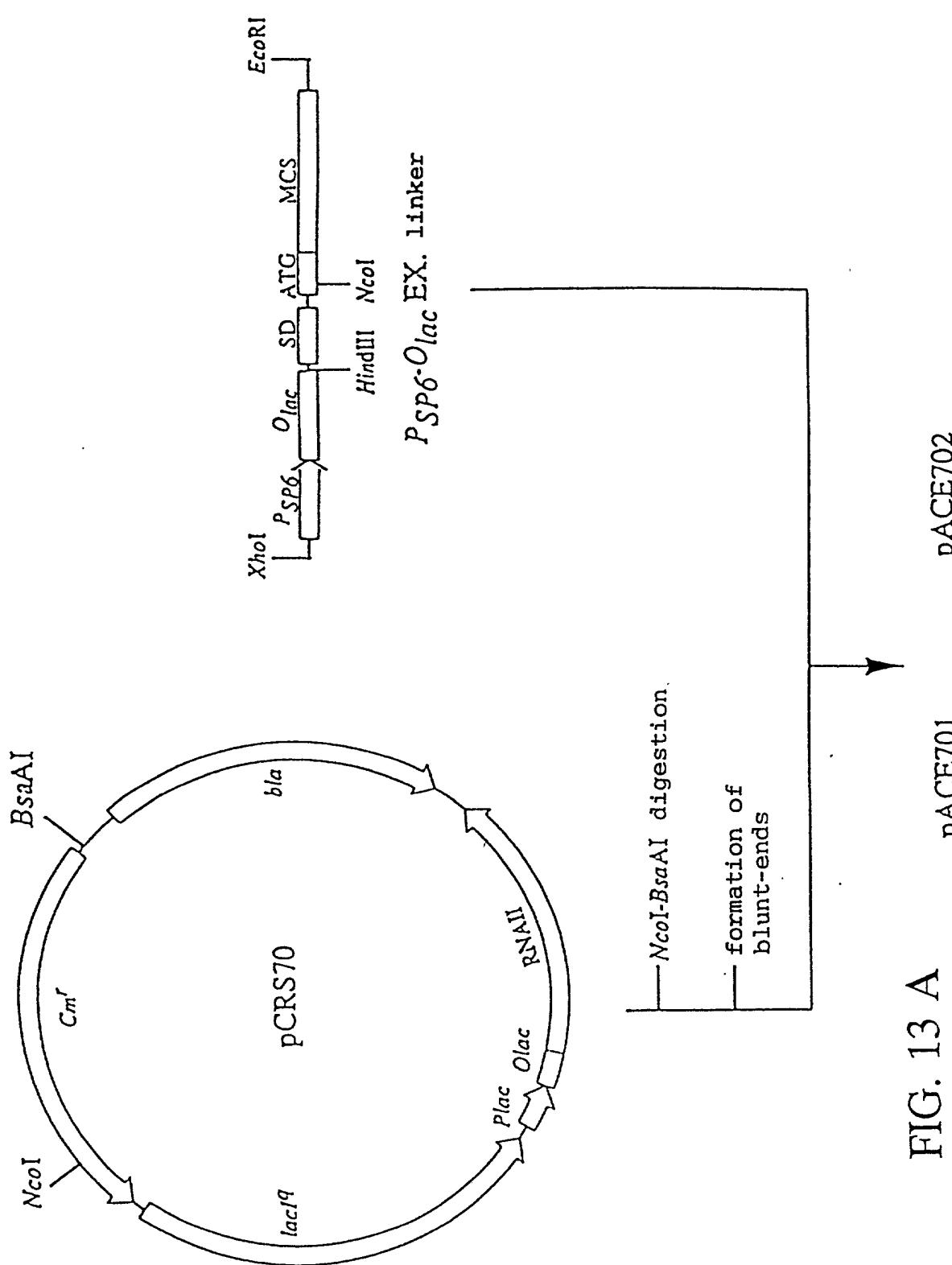


FIG. 13 A

pACE702

pACE701

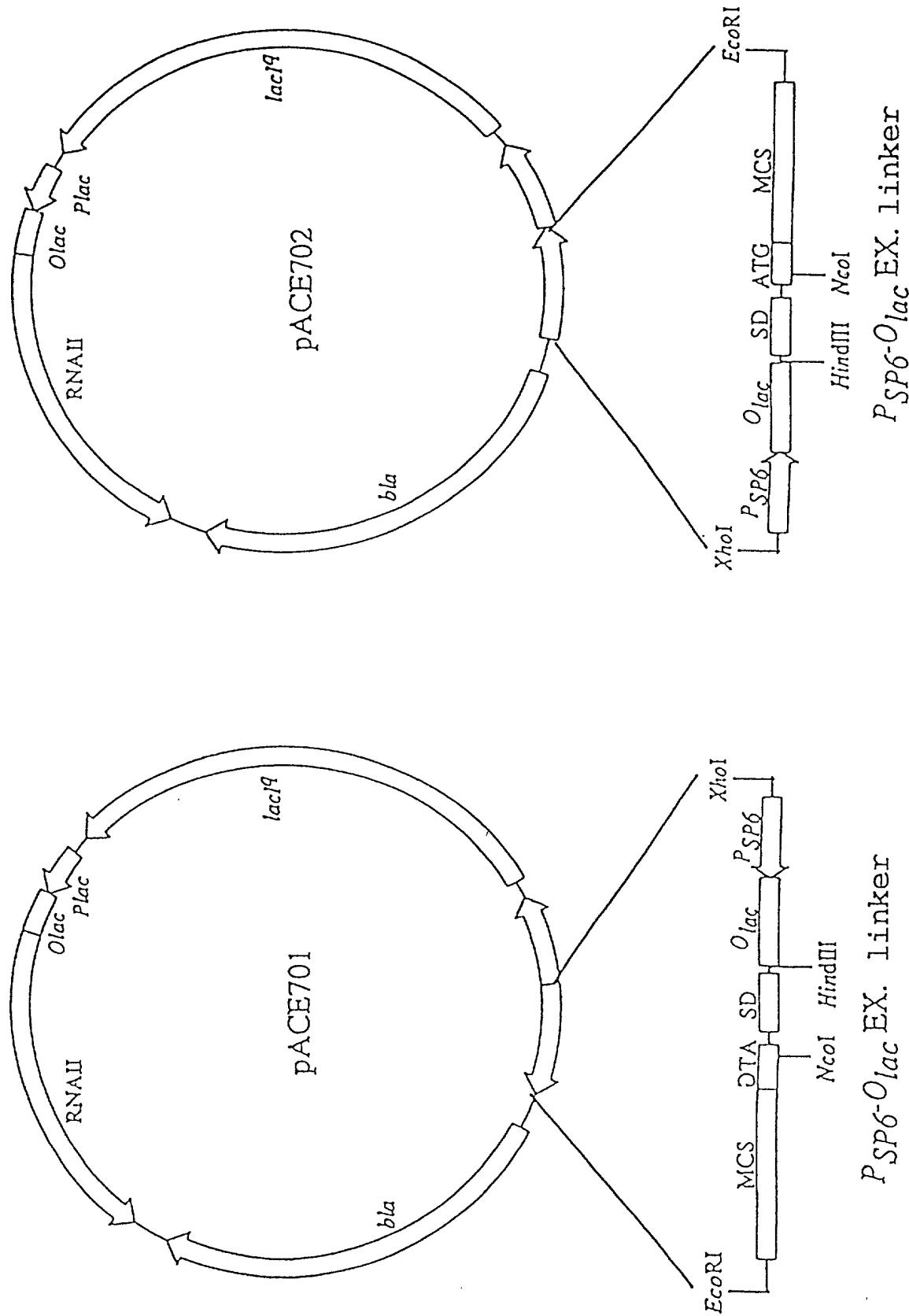


FIG. 13 B

M 1 2 3 4 5 M

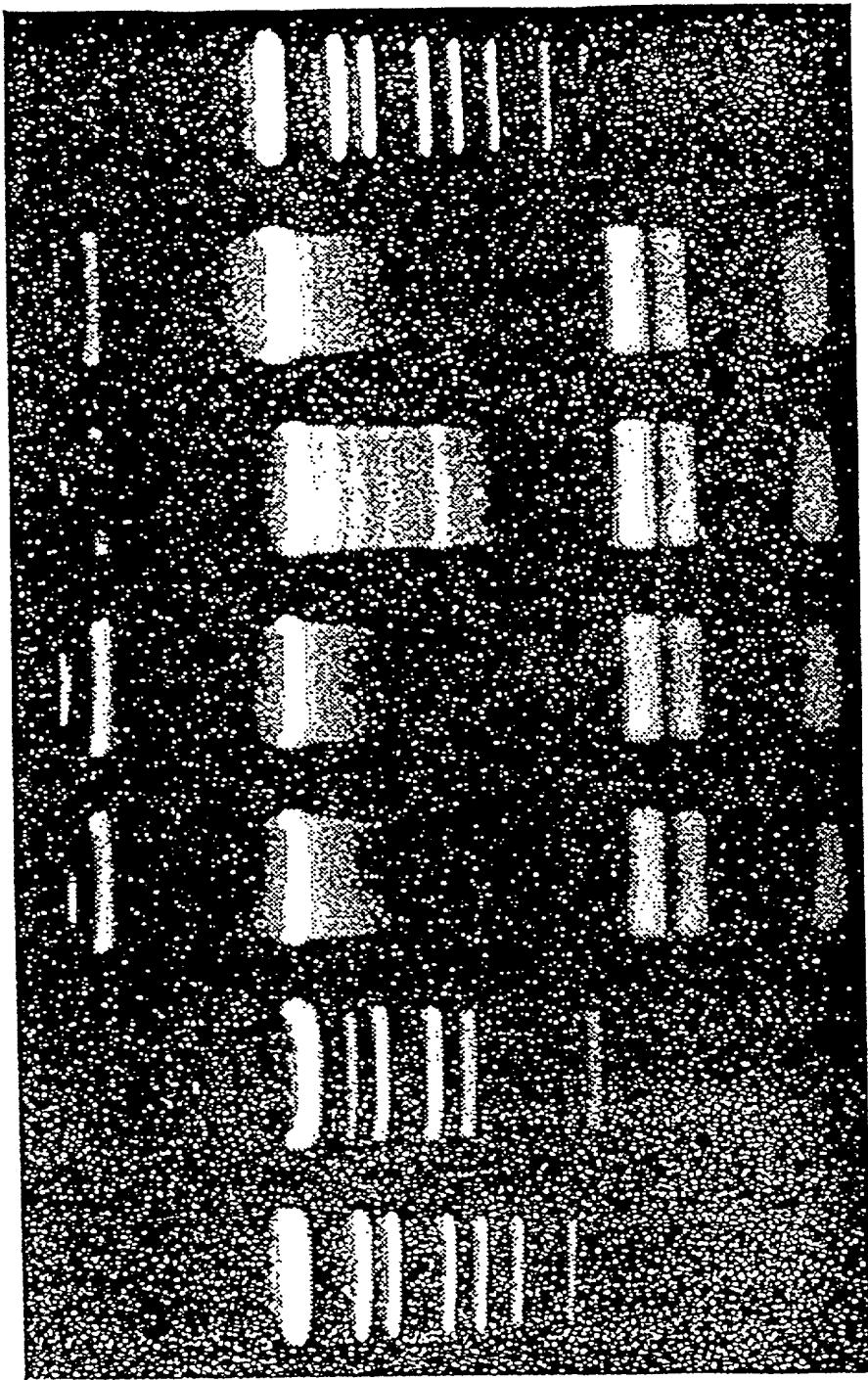
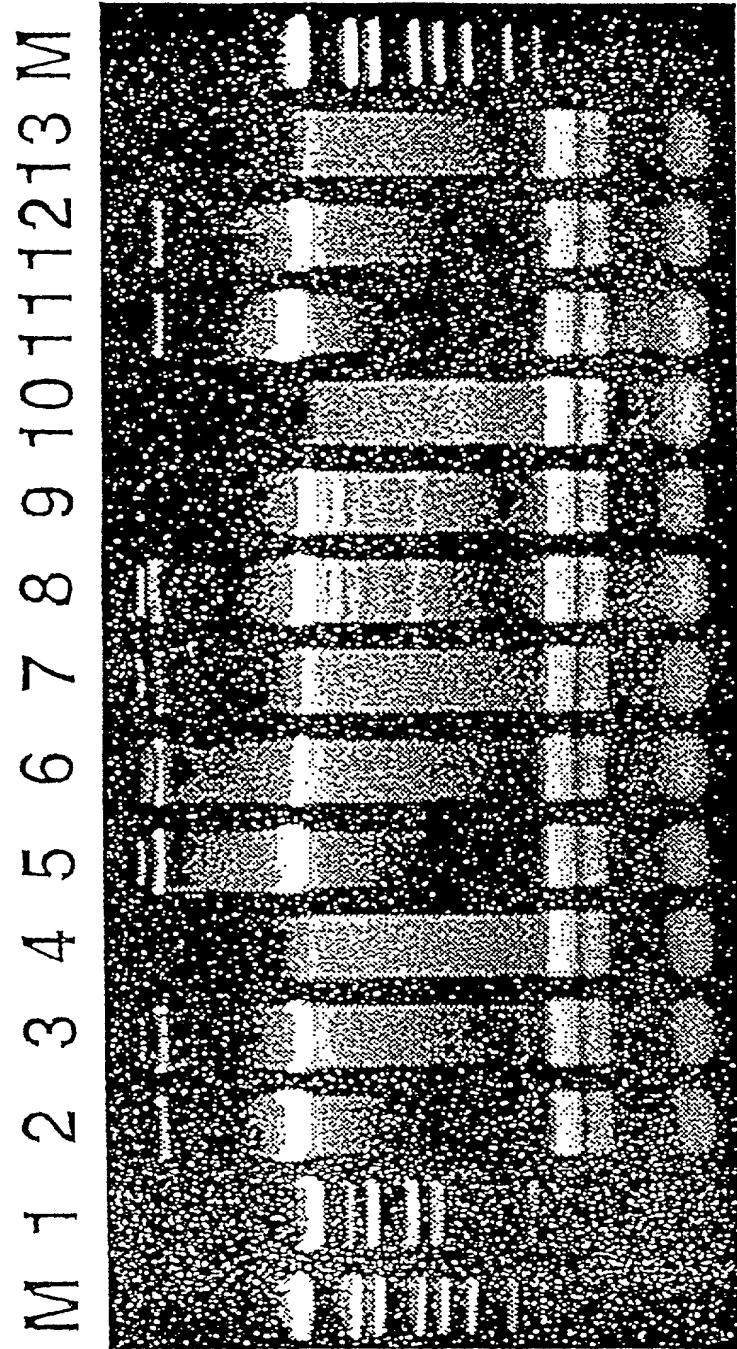


FIG. 14

FIG. 15



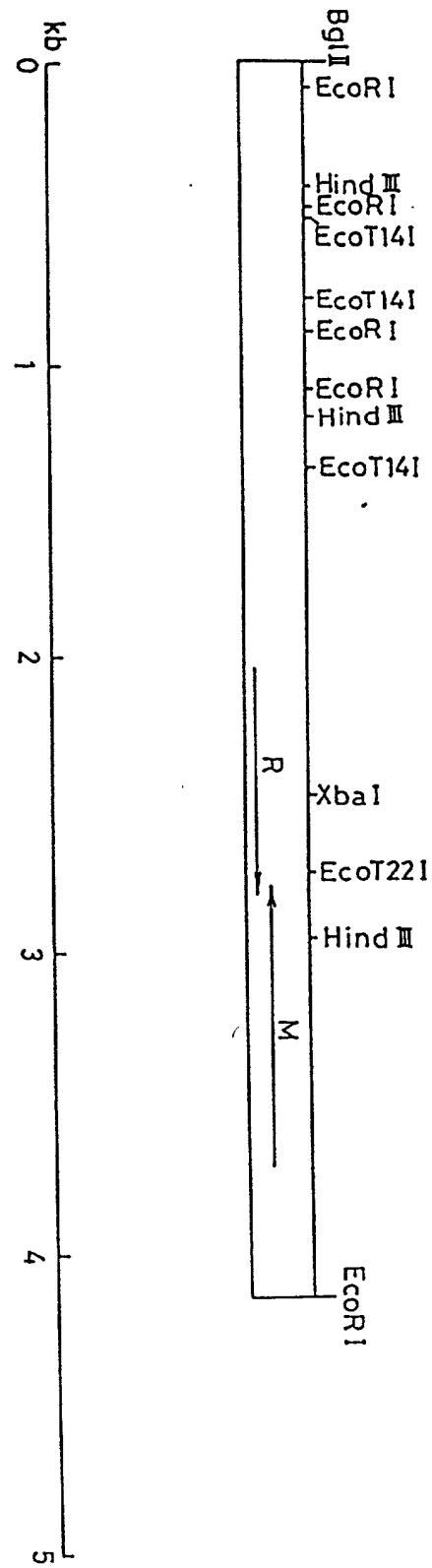


FIG. 16

26 / 26

## BIRCH STEWART, KOLASCH &amp; BIRCH, LLP

COMBINED DECLARATION AND POWER OF ATTORNEY  
FOR PATENT AND DESIGN APPLICATIONS

ATTORNEY DOCKET NO.

1422-319P

PLEASE NOTE:  
YOU MUST  
COMPLETE THE  
FOLLOWING:



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PLASMID

Check Box If  
Appropriate -  
For Use Without  
Specification  
Attached

the specification of which is attached hereto unless the following box is checked:

was filed on March 10, 1997 as United States Application Number \_\_\_\_\_ or PCT International Application Number PCT/JP97/00748 and was amended on \_\_\_\_\_

(if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof, or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

## Prior Foreign Application(s)

Priority Claimed

8-85801	Japan	March 13, 1996	<input checked="" type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Month/Day/Year Filed)	Yes	No
8-208897	Japan	July 18, 1996	<input checked="" type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Month/Day/Year Filed)	Yes	No
(Number)	(Country)	(Month/Day/Year Filed)	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Month/Day/Year Filed)	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Month/Day/Year Filed)	<input type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Month/Day/Year Filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number) (Filing Date)

(Application Number) (Filing Date)

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More Than 12 Months (6 Months for Designs) Prior To The Filing Date of This Application:

Country

Application No.

Date of Filing (Month/Day/Year)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Number) (Filing Date) (Status — patented, pending, abandoned)

(Application Number) (Filing Date) (Status — patented, pending, abandoned)

\*NOTE: Must be completed.

I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

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PLEASE NOTE:  
YOU MUST  
COMPLETE THE  
FOLLOWING:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of First or Sole Inventor:

Insert Name of Inventor

Insert Date This Document Is Signed

Insert Residence

Insert Citizenship

Insert Post Office Address

Full Name of Second Inventor, if any:

see above

Full Name of Third Inventor, if any:

see above

Full Name of Fourth Inventor, if any:

see above

Full Name of Fifth Inventor, if any:

see above

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12-1-510, Nishishibukawa 2-chome, Kusatsu-shi, Shiga 525 Japan			
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Harumi	UENO	<i>Harumi Ueno</i>	October 1, 1997
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7-19, Beppo 2-chome, Otsu-shi, Shiga 520 Japan			
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\*Note: Must be completed — date this document is signed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: SAGAWA, HIROAKI  
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(ii) TITLE OF INVENTION: PLASMID

(iii) NUMBER OF SEQUENCES: 33

(iv) CORRESPONDENCE ADDRESS:

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(D) STATE: VA  
(E) COUNTRY: USA  
(F) ZIP: 22040-0747

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: WEINER, MARC S.  
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(C) REFERENCE/DOCKET NUMBER: 1422-0319P

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 703-205-8000  
(B) TELEFAX: 703-205-8050

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 295 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:

(A) NAME/KEY: Modified-site  
(B) LOCATION: 2  
(D) OTHER INFORMATION: /note= "2=Val or Leu"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Xaa Pro Leu Asp Lys Asp Leu Gln Lys Ala Lys Ile Ser Ile Thr		
1	5	10
Asp Phe Phe Glu Ile Thr Asn Arg Val Leu Asp Tyr Phe Pro Asn Val		
20	25	30
Ile Asn Asn Thr Val Glu Lys Gly Asp Tyr Leu Ile Ser Ser Ser Asn		
35	40	45
Ile Ala Gly Thr Ile Lys Phe Leu Arg Pro Ile Asn Arg Lys Leu Phe		
50	55	60
Ile Gln Glu Lys Lys Val Phe Asn Asp Tyr Phe Gln Lys Leu Ile Ile		
65	70	75
Val Phe Glu Asn Ile Arg Asn Lys Lys Thr Val Thr Glu Glu Asp Lys		
85	90	95
Ile Ile Ile Asp Arg Val Ile Tyr Thr Ile Gln Gln Ser Ile Gly Ile		
100	105	110
Gly Leu Asp Leu Met Val Asn Gln Asn Ser Ala Arg Lys His Val Gly		
115	120	125
Asn Arg Phe Glu Glu Leu Ile Arg Val Ile Phe Thr Glu Ile Ser Val		
130	135	140
Ser Asn Lys Arg Thr Val Leu Gln Ile Pro Tyr Glu Thr Asp Glu Gly		
145	150	155
160		
Gln Lys Ile Tyr Lys Cys Glu Asn Asp Leu Ile Ile Ser Pro Phe Glu		
165	170	175
Asn Val Glu Ser Thr Asn Lys His Leu Asp Glu Asn Glu Ile Val Val		
180	185	190
Ser Ile Lys Thr Thr Ser Lys Asp Arg Met Gly Lys Met Phe Ile Asp		
195	200	205
Lys Ile Leu Leu Glu Arg Phe Val Lys His Pro Gln Lys Val Ile Gly		
210	215	220
Ile Phe Leu Asn Asp Val Gln Arg Lys Glu Asp Asn Asn Ile Ser Phe		
225	230	235
240		
Thr Leu Val Ser Gly Leu Phe Met Val Tyr Thr Lys Phe Leu Thr Thr		
245	250	255
Leu Glu Gly Ile Tyr Tyr Leu Asp Pro Pro Pro Asn Ala Leu Lys Leu		
260	265	270
Pro Tyr Ser Asn His Met Lys Arg Phe Ser Asp Leu Ile Thr Glu Asp		
275	280	285
Leu Glu Lys Leu Phe Ser Ser		
290	295	

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 885 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGSTACAC TGGATAAAGA TTTACAAAAA GCAAAGATT CAATTACTGA	60
TTTTTTGAA	
ATTACAAATA GAGTTTACA TTATTCccc AATGTAATCA ATAATACAGT	120
TGAAAAAGGA	
GATTATTAA TATCCTCATC AAATATTGCT GGAACAATAA AATTCTAACG ACCAATCAAT	180
AGAAAAGTTAT TTATTCAGGA AAAAAAAAGTT TTCAATGATT ATTTCAAAA ACTGATTATA	240
GTaaaaaaaATATAAGGAA CAAAAAAACT GTAACAGAGG AAGATAAAAT TATTATTGAT	300
AGGGTAATTT ACACAATACA GCAATCTATT GGAATTGGTT TAGATTAAAT GGTTAATCAA	360
AATAGTGCTA GAAAGCACGT TGGTAACCGA TTTGAAGAAT TAATTAGAGT CATTTCACA	420
GAAATATCAG TATCGAATAA AAGAACTGTA TTACAAATTC CATATGAAAC TGATGAAGGA	480
CAGAAAATTT ACAAATGCGA GAATGACCTC ATTATTTCTC CTTTGAAAA TGAGAATCT	540
ACAAACAAAC ATCTAGATGA AAATGAGATT GTGTTCAA TAAAGACAAC ATCAAAAGAT	600
AGGATGGAA AAATGTTTAT AGATAAAATT TTACTTGAAA GGTTGTTAA ACACCCTCAA	660
AAAGTTATAG GGATTTCCCT CAATGATGTA CAAAGAAAAG AAGACAACAA TATCAGCTTT	720
ACACTTGTTC CAGGATTATT TATGGTGTAT ACTAAATTCT TAACTACTCT TGAAGGGATC	780
TATTATTTAG ATCCACCACC TAATGCATTG AACTACCCT ATTCTAATCA TATGAAAAGA	840
TTTCAGATT TAATTACAGA AGACCTGAA AAATTATTCT CCTCT	885

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 215 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
 (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TATGGATATG TTCATAAACCA CGCATGTAGG CAGATAGATC TTTGGTGTG AATCGCAACC	60
AGTGGCCTTA TGGCAGGAGC CGCGGATCAC CTACCATCCC TAATGACCTG CAGGCATGCA	120

AGCTTGCATG CCTGCAGGTC ATTAGGTACG GCAGGTGTGC TCGAGGCGAA GGAGTGCCTG	180
CATGCGTTTC TCCTTGGCTT TTTTCCTCTG GGACA	215

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 215 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATGTCCAG AGGAAAAAAG CCAAGGAGAA ACGCATGCAG GCACTCCTTC GCCTCGAGCA	60
CACCTGCCGT ACCTAATGAC CTGCAGGCAT GCAAGCTTGC ATGCCTGCAG GTCTTCTAGGG	120
ATGGTAGGTG ATCCGCGGCT CCTGCCATAA GGCCACTGGT TGCGATTAC AACCAAAGAT	180
CTATCTGCCT ACATGCGTGT TTATGAACAT ATCCA	215

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGATCTAGAG CAAACAAAAA AACCAACCG	28
---------------------------------	----

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGTCTAGATC CCAGAGGAAA AAAG

24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTCGAGATT AGGTGACACT ATAGAACACG GAATTGTGAG CGGATAACAA TTCCAAGCTT

60

CACAGGAAAC AGACCATGGC TTAAGTAACT AGTGAATTG

100

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGAATTCACT AGTTACTTAA GCCATGGTCT GTTTCCTGTG AAGCTTGAA TTGTTATCCG

60

CTCACAAATTC CGTATTCTAT AGTGTACCT AAATCTCGAG

100

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AATCCCAGG AACGCTACGA ATCTCTG

27

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCGGCCATGG TTATTTTGA CACCAGACC

29

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TAACTTGAAT CCATGGGTTC TCACCG

26

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TACTCAGTAG CCATGGCTCT CATAGACCG

29

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 308 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met Asn Glu Ile Ala Phe Asp Asn Tyr Ser Tyr Ile Pro Lys Leu Lys  
1 5 10 15

Leu Tyr Ser Glu Ile Glu Leu Lys Pro Phe Phe Ile Ser Lys Asn Gly  
20 25 30

Ser Leu Phe Asn Val Asp Ala Ile Asp Phe Leu Arg Lys Leu Glu Ser  
35 40 45

Asn Ser Val Asp Leu Ile Phe Ala Asp Pro Pro Tyr Asn Ile Lys Lys  
50 55 60

Ala Glu Trp Asp Ile Phe Ser Ser Gln Asn Glu Tyr Leu Glu Trp Ser  
65 70 75 80

Lys Glu Trp Ile Met Glu Ala His Arg Val Leu Lys Asp Asn Gly Ser  
85 90 95

Leu Tyr Val Cys Gly Phe Ser Glu Ile Leu Ala Asp Ile Lys Phe Ile  
100 105 110

Thr Ser Lys Tyr Phe His Ser Cys Lys Trp Leu Ile Trp Phe Tyr Arg  
115 120 125

Asn Lys Ala Asn Leu Gly Lys Asp Trp Gly Arg Ser His Glu Ser Ile  
130 135 140

Leu Leu Leu Arg Lys Ser Lys Asn Phe Ile Phe Asn Ile Asp Glu Ala  
145 150 155 160

Arg Ile Pro Tyr Asn Glu His Thr Val Lys Tyr Pro Gln Arg Thr Gln  
165 170 175

Ala Glu Ser Ser Gln Tyr Ser Asn Ser Lys Lys Gln Tyr Ile Trp Glu  
180 185 190

Pro Asn Pro Leu Gly Ala Lys Pro Lys Asp Val Leu Glu Ile Pro Thr  
195 200 205

Ile Ser Asn Gly Ser Trp Glu Arg Ser Ile His Pro Thr Gln Lys Pro  
210 215 220

Val Glu Leu Leu Lys Lys Ile Ile Leu Ser Ser Ser Asn Lys Asp Ser  
225 230 235 240

Leu Ile Leu Asp Pro Phe Gly Gly Ser Gly Thr Thr Tyr Ala Val Ala  
245 250 255

Glu Ala Phe Gly Arg Lys Trp Ile Gly Thr Glu Leu Asp Lys Asn Tyr  
260 265 270

Cys Leu Glu Ile Gln Lys Arg Leu Lys Asp Glu Ser Met Ile Asn Arg

275

280

285

Ile	Phe	Ser	Gly	Asp	Asp	Asp	Ser	Asn	Ser	Gln	Asn	Arg	Arg	Lys	Lys
290							295					300			
Leu	Arg	Gly	Glu												
305															

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 924 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTGAATGAAA TAGCGTTGA TAATTACAGT TATATACCAA AATTAAAACT TTATTCGGAA	60
ATCGAGCTTA AACCACTTTT TATTTCAAAA AACGGTTCAC TTTTCAATGT TGATGCTATT	120
GATTTTTAA GAAAATTAGA GAGTAATTCT GTGGATTAA TTTTGCGAGA TCCACCTTAT	180
AACATTAAAA AGGCAGAGTG GGATATTTT TCTTCTCAAA ATGAATATCT CGAATGGAGT	240
AAAGAACATGGA TAATGGAAGC TCATAGAGTT TTAAAGATA ATGGCAGTTT ATATGTTGT	300
GGCTTTTCAG AAATTCTGGC AGACATAAAA TTTATCACTT CAAAATATTT TCACAGTTGT	360
AAATGGTTGA TTTGGTTCTA TAGAAACAAG GCAAATTTAG GTAAAGATTG GGGACGTTCA	420
CACGAAAGTA TACTGTTATT AAGAAAATCT AAAAATTAA TTTTAATAT TGATGAGGCA	480
CGAATCCCCT ATAATGAGCA TACAGTTAA TATCCACAAA GAACCCAGGC CGAATCTTCG	540
CAATATTCGA ACTCAAAAAA GCAATATATT TGGGAGCCAA ACCCATTAGG AGCTAAGCCA	600
AAAGATGTTT TGGAGATTCC CACAATTCA AATGGTTCTT GGGAAAGAAG TATTCAACCCT	660
ACGCAAAAGC CAGTAGAATT GCTTAAAAAA ATAATTTAT CTTCATCTAA TAAAGATAGT	720
TTAATTCTTG ATCCATTGG TGGTTCGGGA ACTACATATG CTGTTGCGGA AGCTTTGGC	780
AGAAAATGGA TTGGAACAGA GTTAGATAAA ATTATTGTC TGGAAATTCA AAAGCGATTG	840
AAAGACGAAA GTATGATCAA CAGGATTTC TCAGGCGATG ATGATTCAA TTCTCAAAAT	900
AGAAGAAAAA AATTAAGAGG AGAA	924

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCGAGATTAA GGTGACACTA TAGAATACA

29

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGCTTGTATT CTATA GTGTC ACCTAAATC

29

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 54 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCGAGATTAA GGTGACACTA TAGAATACGG AATTGTGAGC GGATAACAAT TCCA

54

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 54 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AGCTTGGAAAT TGTTATCCGC TCACAATTCC GTATTCTATA GTGTCACCTA AATC

54

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met Leu Pro Leu Asp Lys Asp Leu Gln Lys Ala Lys Ile Ser Ile Thr  
1 5 10 15

Asp Phe Phe Glu  
20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic DNA"

(ix) FEATURE:

- (A) NAME/KEY: primer\_bind
- (B) LOCATION: 1..23
- (D) OTHER INFORMATION: /note= "6, 9, 12 = inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATGTTNCCNY TNGAYAARGA YYT

23

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic DNA"

(ix) FEATURE:

- (A) NAME/KEY: primer\_bind  
(B) LOCATION: 1..23  
(D) OTHER INFORMATION: /note= "9 = inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AAGGATTTCNC ARAARGCNAAA RAT

23

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TAAATCTAAA CCAATTCCAA TAGATTGCTG

30

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TAAATCTAAA CCAATTCCAA TAGATTGCTG

30

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GAACTGTATT ACAAAATTCCA TATGAAAATCG

30

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GACAGAAAAT TTACAAATGC GAGAATGACC

30

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1588 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCATGGCACCA CGTTCAAAA AAGAAATCCT CGAAGTCAAA TATGATGAGA AAAACATCTC	60
AGACATCCTG CATATGACGG TGGATGAAGC ATTGGAATT TTCTCGGAAA ATCACGAAGA	120
AAAAATTGTA ACCAAACTAA AACCTTGCA GGACGTTGGT TTGGGTTATC TTCAAGTTAGG	180
CCAGTCCTCC TCTACTCTT CCGGCGGTGA AGCCCAAAGA GTGAAGCTCG CCTCTTCCT	240
TGTGAAAGGT GTAACGACGG AAAAAACGTT ATTTGTTTT GATGAACCAT CAACAGGATT	300
ACATTTCAC GACATTCAAA AATTACTGAA ATCACTTCAG GCACTGATAG AATTAGGGCA	360
TTCGGTTGTA GTGATTGAGC ATCAGCCGG A TATTATCAA TGCGCCGATT ACATCATCGA	420
TGTCGGACCC AATGCCGGAA AATACGGTGG CGAAATTGTT TTCACAGGAA CTCCGGAAGA	480
TTTGGTAAA GAGAAAAAGT CGTTACAGG GAAGTATATT AAGGAGAAGT TAAAGTAATT	540
TATTTATATT TGAAGTTATG CTACCACTGG ATAAAGATT ACAAAAGCA AAGATTTCAA	600
TTACTGATT TTTGAAATT ACAAAATAGAG TTTAGATTA TTTCCCCAAT GTAATCAATA	660
ATACAGTTGA AAAAGGAGAT TATTAATAT CCTCATCAA TATTGCTGGA ACAATAAAAT	720
TCCTAAGACC AATCAATAGA AAGTTATTTA TTCAGGAAAA AAAAGTTTC AATGATTATT	780

TTCAAAAAC T GATTATAGTT TTTGAAAATA TAAGGAACAA AAAAACTGTA ACAGAGGAAG	840
ATAAAATTAT T ATTGATAGG GTAATTACA CAATACAGCA ATCTATTGGA ATTGGTTAG	900
ATTTAATGGT TAATCAAAAT AGTGCTAGAA AGCACGTTGG TAACCGATT GAAGAATTAA	960
TTAGAGTCAT TTTTACAGAA ATATCAGTAT CGAATAAAAG AACTGTATTA CAAATTCCAT	1020
ATGAAACTGA TGAAGGCACAG AAAATTACA AATGCGAGAA TGACCTCATT ATTTCTCCTT	1080
TTGAAAATGT AGAACATCACA AACAAACATC TAGATGAAAA TGAGATTGTT GTTCAATAA	1140
AGACAAACATC AAAAGATAGG ATGGGAAAAA TGTTTATAGA TAAAATTAA CTTGAAAGGT	1200
TTGTTAAACA CCCTCAAAAAA GTTATAGGGA TTTTCCTCAA TGATGTACAA AGAAAAGAAG	1260
ACAACAATAT CAGCTTACA CTTGTTTCAG GATTATTAT GGTGTATACT AAATTCTTAA	1320
CTACTCTTGA AGGGATCTAT TATTTAGATC CACCACCTAA TGCATTGAAA CTACCATATT	1380
CTAATCATAT GAAAAGATT TCAGATTAA TTACAGAAGA CCTTGAAAAA TTATTCTCCT	1440
CTTAATTTTT TTCTTCTATT TTGAGAATT GAATCATCAT CGCCTGAAAA AATCCTGTTG	1500
ATCATACTTT CGTCTTCAA TCGCTTTGA ATTTCCAGAC AATAATTAA ATCTAACTCT	1560
GTTCCAATCC ATTTCTGCC AAAAGCTT	1588

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
  
- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ATATTGAAAG CCATGGTACC ACTGG	25
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(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
  
- (ii) MOLECULE TYPE: other nucleic acid
  - (A) DESCRIPTION: /desc = "synthetic DNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

## (2) INFORMATION FOR SEQ ID NO:29:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4146 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AGATCTGGTC ATCCCAAACA AAAATCTTC GGTTTACGAA GATGCAGTCG CTTCTGGAA	60
AGGCAGAAAGT ATGAGCGAAT GGAAAAAGGA ATTCACTAAA AAAGCCAAAG ATTCCCAAT	120
TCACAAGCCT TATCATCAAC TCACAAAAGA GCAGAACAG TTCCTTGGA AAGGCGATAA	180
AACCAGAAGT TTCCCAAGTA TTGATAATT TTTCAAATG CTTGAAGAGA ATCTTACAA	240
GATCCAATAC CGCGTAATGC TTTCGCGCTA TCGTGGAAA ACACTTGCC CCGATTGCGA	300
AGGATTACGA TTGCGGGAAAG AAACAAGCTG GGTGAAGATT GACGGACACA ACATTCACTC	360
TTTGATTGAA TTACCTTGGA ATGAACCTCCT GCCATTGATC AAAAGCTTAA AACTGAACGT	420
CCACGACAGA GAAATTGCCA AACGCCTGAC TTACGAAATC GAAACGAGAT TAGAATTCCCT	480
GACGAAAGTC GGCCTGGAT ATCTGACTTT GAACCGAACCA TCCAACACGC TTTCCGGAGG	540
AGAAAGCCAG AGAATCAATC TGGCGACAGC TTGGGAAGTT CGCTGGTTGG TTCTATTTAT	600
ATTTTGGATG AGCCGAGCAT TGGTCTGCAT TCCCGCGATA CAGAAAATCT GATTGGTGTG	660
CTCAAACAAAC TCCCGGATTT GGAAANTACC GTGATTGTTG TAGAACACGA CGAAGATGTG	720
ATGCTTGGGG CAGNTTACAT TATAGATATT GGCCCNGNAG CGGGCTACCT TGGTGGCGAT	780
CTTGTGTTCA GCGNGGATTA TAAAGAGATG CTGAAGTNNTN ATACTTTAAC CGCAAAATAC	840
CTGAATGGCG AACTGAAAAT AGAAGTTCCT GAAAAACGAA GAAAACCGAA GGAATTCAATC	900
GCAATAAAAG GTGCCGCCA GAATAATTAA AAAAATATTG ACGTTGATGT TCCGTTAGAA	960
TGTCTGACAG TTATCACAGG CGTTCTGGA AGCGGGAAAT CCACTTGAT GAAGGAAGTG	1020
ATGACCAATG CCATCCAGAT CCAACTGGGA ATGGGCGGCA AAAAAGCCGA TTACGATTG	1080
GTGGAATTCC CGAAAAAGCT GATCCAGAAT ATCGAACTGA TTGACCAGAA CCCAATCGGG	1140
AAATCGTCCC GCTCCAACCC CGTGACTTAT CTGAAAGCTT ACGACGATAT CCGGGATCTT	1200
TTTGCAGAAC AAAAATCCGC AAAAATCCAG GGTACAAAC CGAAGCATT CTCCCTCAAT	1260
GTGGATGGCG GAAGATGTGA CGAGTGCAAA GGCGAAGGTA TCATTACCGT ATCAATGCAG	1320

TTTATGGCGG ACATCGAGCT GGAGTGTGAG CATTGCCATG GCACACGTTT CAAAAAAAGAA	1380
ATCCTCGAAG TCAAATATGA TGAGAAAAAC ATCTCAGACA TCCTGCATAT GACGGTGGAT	1440
GAAGCATTGG AATTTTCTC GGAAAATCAC GAAGAAAAAA TTGTAACCAA ACTAAAACCT	1500
TTGCAGGACG TTGGTTTGGG TTATCTTCAG TTAGGCCAGT CCTCCTCTAC TCTTTCCGGC	1560
GGTGAAGCCC AAAGAGTGAA GCTCGCCTCT TTCCCTGTGA AAGGTGTAAC GACGGAAAAA	1620
ACGTTATTTG TTTTGATGA ACCATCAACA GGATTACATT TCCACGACAT TCAAAAATTA	1680
CTGAAATCAC TTCAGGCACT GATAGAATTAA GGGCATTCGG TTGTAGTGAT TGAGCATCAG	1740
CCGGATATTA TCAAATGCGC CGATTACATC ATCGATGTCG GACCCAATGC CGGAAAATAC	1800
GGTGGCGAAA TTGTTTCAC AGGAACCTCG GAAGATTGG TAAAAGAGAA AAAGTCGTTT	1860
ACAGGGAAGT ATATTAAGGA GAAGTTAAAG TAATTTATTT ATATTTGAAG TTATGCTACC	1920
ACTGGATAAA GATTACAAA AAGCAAAGAT TTCAATTACT GATTTTTTG AAATTACAAA	1980
TAGAGTTTA GATTATTCC CCAATGTAAT CAATAATACA GTTGAAAAAG GAGATTATTT	2040
AATATCCTCA TCAAATATTG CTGGAACAAT AAAATTCTA AGACCAATCA ATAGAAAGTT	2100
ATTTATTCAAG GAAAAAAAAG TTTTCAATGA TTATTTCAA AAACGTGATTA TAGTTTTGA	2160
AAATATAAGG AACAAAAAAA CTGTAACAGA GGAAGATAAA ATTATTATTG ATAGGGTAAT	2220
TTACACAATA CAGCAATCTA TTGGAATTGG TTTAGATTAA ATGGTTAACATC AAAATAGTGC	2280
TAGAAAGCAC GTTGGTAACC GATTGAAAGA ATTAATTAGA GTCATTTTA CAGAAATATC	2340
AGTATCGAAT AAAAGAACTG TATTACAAAT TCCATATGAA ACTGATGAAG GACAGAAAAT	2400
TTACAAATGC GAGAATGACC TCATTATTTC TCCTTTGAA AATGTAGAAT CTACAAACAA	2460
ACATCTAGAT GAAAATGAGA TTGTTGTTTC AATAAGACA ACATCAAAAG ATAGGATGGG	2520
AAAAATGTTT ATAGATAAAA TTTTACTTGA AAGGTTGTT AAACACCCCTC AAAAAGTTAT	2580
AGGGATTTC CTCAATGATG TACAAAGAAA AGAAGACAAC AATATCAGCT TTACACTTGT	2640
TTCAGGATTA TTTATGGTGT ATACTAAATT CTTAACTACT CTTGAAGGGAA TCTATTATTT	2700
AGATCCACCA CCTAATGCAT TGAAACTACC ATATTCTAAT CATATGAAAA GATTTTCAGA	2760
TTTAATTACA GAAGACCTTG AAAAATTATT CTCCTCTAA TTTTTTCTT CTATTTGAG	2820
AATTGAAATC ATCATCGCCT GAAAAAATCC TGTTGATCAT ACTTCGTCT TTCAATCGCT	2880
TTTGAATTTC CAGACAATAA TTTTATCTA ACTCTGTTCC AATCCATTCTT CTGCCAAAAG	2940
CTTCCGCAAC AGCATATGTA GTTCCCGAAC CACCAAATGG ATCAAGAATT AAACTATCTT	3000
TATTAGATGA AGATAAAATT ATTTTTTAA GCAATTCTAC TGGCTTTGC GTAGGGTGAA	3060
TACTTCTTTC CCAAGAACCA TTTGAAATTG TGGGAATCTC CAAAACATCT TTTGGCTTAG	3120

CTCCTAATGG	GTTTGGCTCC	CAAATATATT	GCTTTTGA	GTTCGAATAT	TGCGAAGATT	3180
- CGGCCTGGGT	TCTTGTTGGA	TATTTAACTG	TATGCTCATT	ATACGGGATT	CGTGCCTCAT	3240
CAATATTAAA	AATAAAATTT	TTAGATTTTC	TTAATAACAG	TATACTTCG	TGTGAACGTC	3300
CCCAATCTT	ACCTAAATTT	GCCTTGTTC	TATAGAACCA	AATCAACCAT	TTACAACGT	3360
GAAAATATT	TGAAGTGATA	AATTTATGT	CTGCCAGAAT	TTCTGAAAAG	CCACAAACAT	3420
ATAAACTGCC	ATTATCTTT	AAAACTCTAT	GAGCTTCCAT	TATCCATTCT	TTACTCCATT	3480
CGAGATATT	C ATTTGAGAA	GAAAAAATAT	CCCAC TCTGC	CTTTTAATG	TTATAAGGTG	3540
GATCTGAAA	AATTAATCC	ACAGAATTAC	TCTCTAATT	TCTTAAAAAA	TCAATAGCAT	3600
CAACATTGAA	AA GTGAACCG	TTTTTGAAA	TAAAAAATGG	TTTAAGCTCG	ATTTCCGAAT	3660
AAAGTTTAA	TTTTGGTATA	TA ACTGTAAT	TATCAAACGC	TATTCATTC	ACAAATGAAT	3720
CAATCTGCTG	TTGTGTATAA	ACCCTGTAAT	TATTAATAGG	ATGTCTAAA	CTTTGAATT	3780
TTCCAGAATT	ATCCCAC TTT	CCTTAATGTC	TCAGAGTTAA	CATCTAATAA	TTTCGCCGCT	3840
TCTTTATTG	ATAAATAATC	ATCCATATCT	TACACAACAT	TACACAAGTT	TATACAGCAA	3900
ATATAAATAT	TTTTTATACA	TTGTAAAAAT	TTTATTTACT	TTTATTTGT	TCAATTGTCT	3960
CAATAAATAG	TTAATCGAAA	TACATTTGA	ATATGATAAA	ATTGACTCCA	ACAAATCTAA	4020
CACAATGACA	TTAAAACCAA	TAAAAACCGA	AGAAGATTAC	AATCAGGTTT	TAGAAAGACT	4080
TTCACAAATT	TTCGACGCTA	AACCAAATAC	CAAAGATGGA	GATGAATTGG	GAAATCTTGG	4140
GAATTC						4146

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATTTAGGTGA CACTATAGAA TAC

23

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 49 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AAGCTCGAGT CTGATGACGA AGCTTGACTG ACTGAGATCA GCTTGCAAC

49

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTCGAGATT AGGTGACACT ATAGAATACA AGCTT

35

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTCGAGATT AGGTGACACT ATAGAATACG GAATTGTGAG CGGATAACAA TTCCAAGCTT

60